M19-110

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : A61K A2

(11) International Publication Number: **WO 00/49991**

(43) International Publication Date: 31 August 2000 (31.08.00)

(21) International Application Number: PCT/US99/14751

(22) International Filing Date: 29 June 1999 (29.06.99)

(30) Priority Data:
09/256,484 23 February 1999 (23.02.99) US

(71) Applicant: PHAIRSON MEDICAL INC. [US/US]; 602 The Chambers, Chelsea Harbour, London SW10 0XF (GB).

(72) Inventors: FRANKLIN, Richard; 39 Dymock Street, London SW6 3ED (GB). COWLING, Didier; Copperfield Park Close, Esher, Surrey KT10 8LG (GB). HUBBELL, Jeffrey, A.; Zürich (CH).

(74) Agents: BLOOM, Allen et al.; Dechert Price & Rhoads, P.O. Box 5218, Princeton, NJ 08054-5218 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: TREATMENT OF TRAUMA

(57) Abstract

Provided is a method of treating an area affected by a trauma, such as a corneal wound or internal trauma, comprising administering to the affected area a trauma treating effective amount of a composition comprising a microgel comprising a crosslinked polyanionic polymer.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

TREATMENT OF TRAUMA

The present invention relates to the use of a microgel material with or without certain enzymes, for treating wounds, such as corneal ulcerations, and internal trauma. The present invention further relates to the use of an enzyme in any pharmaceutically acceptable carrier for the treatment of corneal wounds and prevention of adhesions.

Various treatments for tissue trauma or wounds are known in the art. However, particularly with regard to corneal ulcerations or abrasions, for example, there are few, if any, non-invasive procedures that effectively and economically reduce or prevent permanent damage to the cornea. Accordingly, a not uncommon result of such injuries to the cornea is partial or total blindness in the affected eye. New non-invasive treatments would be welcome.

The formation of adhesions on internal organs and tissues, such as between the body wall and internal organs, following internal surgery is a significant medical problem. New methods and treatments for treating internal trauma to suppress adhesion formation are needed.

Other wounds for which improved treatments have been obtained include cutaneous wounds such as decubitus ulcers, venous ulcers, burns, or pressure sores.

SUMMARY OF THE INVENTION

The present invention relates, among other things to a method of treating an area affected by trauma, in particular corneal wounds and internal trauma, that includes administering to the affected area a trauma treating effective amount of a composition including a microgel comprising a polyanionic polymer.

In one aspect, the method deals with treatment of corneal ulcers, including those that accompany infection associated with a pathogen, corneal abrasions, and chemical and physical insult to the cornea. In another aspect, the method deals with treatment of internal surgical wounds.

In one embodiment a method provides for treating trauma to membranes, for example an incision through membranes that cover internal organs and tissues, and that cover cavities that contain organs and tissues. In particular embodiments, the membranes are serous membranes such as the peritoneum, the pericardium, the epicardium, and the pleura. In other embodiments the method treats trauma to the epithelia, which includes endothelia, and the meninges. In yet another embodiment, the method provides for treatment of internal trauma to tendons, tendon sheaths, nerves, and nerve sheaths.

In a preferred embodiment, the method is directed at treatment of internal trauma that is susceptible to giving rise to adhesions by application of an effective amount of a composition including a microgel to the area affected by internal trauma.

In one embodiment, the composition including a microgel used in the practice of the method can also includes a protease, such as a serine protease. In a preferred embodiment, the protease has an activity of at least two of a chymotrypsin, trypsin, collagenase, and elastase activity. In a particularly preferred embodiment that is a treatment for a corneal wound that can be a corneal ulcer, a corneal

abrasion, or a chemical or physical insult to the cornea, the protease is a multifunctional enzyme that is (a) a first enzyme and has at least about 60% sequence similarity with a reference sequence which is AA64-300 of SEQ ID NO:2 or AA1-300 of SEQ ID NO:2 or a sequence differing from these by at least one of the residue differences found in SEQ ID NO:4, 6, 8, 10, or 12 or (b) a second enzyme which is *Panaeus vanameii* 1, *Panaeus vanameii* 2, *Panaeus monodon* chymotryptic-1, *Panaeus monodon* tryptic, *Panaeus monodon* chymotryptic-2, *Uca pugilator* enzyme I, *Uca pugilator* enzyme II, Kamchatka crab IA, Kamchatka crab IIA, Kamchatka crab IIB, Kamchatka crab IIC, Crayfish protease 1, Salmon enzyme 1, Atlantic cod I, or Atlantic cod II.

The method of the present invention can be practiced with microgels that include a crosslinked polyanionic polymer wherein the crosslinked polyanionic polymer is made by polymerization of one or more ethylenically unsaturated crosslinking agents and one or more ethylenically unsaturated compounds at least one of which has:

i) one or more functional groups that can be titrated with base to form negatively charged functional groups, or

ii) one or more precursor groups that are precursors of the functional groups that can be titrated with base; which precursor groups are converted to the functional groups. Preferred functional groups are selected from $-C(O)OR^4$; $-S(O_2)OR^4$; or $-S(O)OR^4$; wherein R^4 is hydrogen. Preferred precursor groups are selected from $-C(O)R^4$, $-S(O_2)OR^4$, or $-S(O)OR^4$; wherein R^4 is independently C_1 - C_6 normal or branched alkyl, phenyl, or benzyl.

The microgels with which the present method is practiced comprise polyanionic polymers. Preferred polyanionic polymers can be made by polymerization of a combination of one or more ethylenically unsaturated crosslinking agents and one or more ethylenically unsaturated monomers, at least one of which can be represented by the following structure:

$$(R^3)(R^2)C=C(R^1)\text{-}X\text{-}Y$$

wherein:

Y is $-C(O)OR^4$; $-S(O_2)OR^4$; or $-S(O)OR^4$; wherein R^4 is hydrogen, C_1 to C_6 normal or branches alkyl, phenyl, or benzyl,

X is a direct bond; a straight or branched alkylene group having two to six carbon atoms, one or more of which can be replaced by O, S, or N heteroatoms, provided that there is no heteroatom in a position α or β to Y; phenylene; a five or six membered heteroarylene having up to three heteroatoms independently selected from O, S, and N, provided that neither Y or $R^3R^2C=C(R^1)$- is bonded to a heteroatom; and

R^1, R^2, and R^3 are independently selected from, hydrogen, C_1-C_6 alkyl, carboxy, halogen, cyano, isocyanato, C_1-C_6 hydroxyalkyl, alkoxyalkyl having 2 to 12 carbon atoms, C_1-C_6 haloalkyl, C_1-C_6 cyanoalkyl, C_3-C_6 cycloalkyl, C_1-C_6 carboxyalkyl, aryl, hydroxyaryl, haloaryl, cyanoaryl, C_1-C_6 alkoxyaryl, carboxyaryl, nitroaryl, or; and a group -X-Y; wherein C_1-C_6 alkyl or C_1-C_6 alkoxy groups are either linear or branched and up to Q-2 carbon atoms of any C_3-C_6 cycloalkyl group, wherein Q is the

total number of ring carbon atoms in the cycloalkyl group, are independently replaced with O, S, or N heteroatoms; with the proviso that neither doubly-bonded carbon atom is directly bonded to O or S; and wherein aryl is phenyl or a 5 or 6 membered heteroaryl group having up to three heteroatoms selected from the group consisting of O, S, and N. In preferred embodiments, R^1, R^2 and R^3 are independently hydrogen or C_1-C_3 alkyl and X is a direct bond or C_1-C_3 alkylene. In yet more preferred embodiments, R^2 and R^3 are hydrogen; R^1 is either hydrogen or methyl, and X is a direct bond. In still more preferred embodiments, Y is $-C(O)OR^4$, where R^4 is hydrogen or methyl, preferably hydrogen.

In particularly preferred embodiments the mole fraction of double bonds attributable to the ethylenically unsaturated crosslinking agent in the combination of crosslinking agent and ethylenically unsaturated monomer from which the polyanionic polymers of the microgels used in the present method can be made, is 0.02 or less, preferably 0.01 or less. Preferred ethylenically unsaturated crosslinking agents are the triallyl ether of sucrose or, more preferably, the trially ether of pentaerythritol. The microgel used in preferred embodiments of the present invention has a macroviscosity-to-microviscosity ratio of the microgel 10,000 or less.

In another embodiment, the present invention provides a method for treating trauma susceptible to giving rise to the formation of adhesions by administering to the area affected by such trauma with an effective amount of a composition that includes a microgel comprising a crosslinked polyanionic polymer. When the trauma is to the peritoneum, 200 to 300 ml of microgel containing 0.5% to 2.5% by weight crosslinked polyanionic polymer is a typical effective amount, but the practitioner will know to modify this amount according to the location, size, and severity of the trauma.

In yet another embodiment, the invention provides a method of treating a wound comprising administering to the affected area an effective amount of a composition comprising a microgel comprising a crosslinked polyanionic polymer made by polymerization of one or more ethylenically unsaturated crosslinking agents and one or more ethylenically unsaturated compounds at least one of which has: i) one or more functional groups that can be titrated with base to form negatively charged functional groups, or ii) one or more precursor groups that are precursors of the functional groups that can be titrated with base; which precursor groups are converted to the functional groups; and wherein at least one of the following is true: the mole fraction of total ethylenic double bonds in the combination from which the crosslinked polyanionic polymer is made that is contributed by the ethylenically unsaturated crosslinking agent is 0.02 or less, and the ratio of macroviscosity of the microgel to the microviscosity of the microgel is 10,000 or less. Preferably, the functional groups are selected from $-C(O)OR^4$; $-S(O_2)OR^4$; or $-S(O)OR^4$; wherein R^4 is hydrogen, and the precursor groups are selected from $-C(O)R^4$, $-S(O_2)OR^4$, or $-S(O)OR^4$; wherein R^4 is independently C_1 - C_6 normal or branched alkyl, phenyl, or benzyl.

The preferred method for treating wounds employs a microgel that includes at least one crosslinked polyanionic polymer made from a combination having at least one ethylenically unsaturated monomer that can be represented by the structure

$(R^3)(R^2)C=C(R^1)$-X-Y

wherein:

Y is $-C(O)OR^4$; $-S(O_2)OR^4$; or $-S(O)OR^4$; wherein R^4 is hydrogen, C_1-C_6 alkyl, phenyl, or benzyl;

X is a direct bond; a straight or branched alkylene group having two to six carbon atoms, one or more of which can be replaced by O, S, or N heteroatoms, provided that there is no heteroatom in a position α or β to Y; phenylene; a five or six membered heteroarylene having up to three heteroatoms independently selected from O, S, and N, provided that neither Y or $R^3R^2C=C(R^1)$- is bonded to a heteroatom; and

R^1, R^2, and R^3 are independently selected from, hydrogen, C_1-C_6 alkyl, carboxy, halogen, cyano, isocyanato, C_1-C_6 hydroxyalkyl, alkoxyalkyl having 2 to 12 carbon atoms, C_1-C_6 haloalkyl, C_1-C_6 cyanoalkyl, C_3-C_6 cycloalkyl, C_1-C_6 carboxyalkyl, aryl, hydroxyaryl, haloaryl, cyanoaryl, C_1-C_6 alkoxyaryl, carboxyaryl, nitroaryl, or; and a group -X-Y; wherein C_1-C_6 alkyl or C_1-C_6 alkoxy groups are either linear or branched and up to Q-2 carbon atoms of any C_3-C_6 cycloalkyl group, wherein Q is the total number of ring carbon atoms in the cycloalkyl group, are independently replaced with O, S, or N heteroatoms; with the proviso that neither doubly-bonded carbon atom is directly bonded to O or S and wherein aryl is phenyl or a 5 or 6 membered heteroaryl group having up to three heteroatoms selected from the group consisting of O, S, and N. In certain embodiments, R^1, R^2, R^3, are hydrogen, X is a direct bond, and Y is COOH. In preferred embodiments the microgel is in a composition that also contains a multifunctional protein.

In yet another embodiment, the present invention provides a method of treating a corneal wound, particularly a corneal ulcer, including that associated with infection, a corneal abrasion, or a physical or chemical insult susceptible to giving rise to a corneal ulcer, comprising administering to an affected area an effective amount of a composition comprising a protease that has an activity comprising at least two of a chymotrypsin, trypsin, collagenase, and elastase activity. Preferably, the protease is a multifunctional enzyme that is (a) a first enzyme and has at least about 60% sequence similarity with a reference sequence which is AA64-300 of SEQ ID NO:2 or AA1-300 of SEQ ID NO:2 or a sequence differing from these by at least one of the residue differences found in SEQ ID NO:4, 6, 8, 10, or 12 or (b) a second enzyme which is *Panaeus vanameii* 1, *Panaeus vanameii* 2, *Panaeus monodon* chymotryptic-1, *Panaeus monodon* tryptic, *Panaeus monodon* chymotryptic-2, *Uca pugilator* enzyme I, *Uca pugilator* enzyme II, Kamchatka crab IA, Kamchatka crab IIA, Kamchatka crab IIB, Kamchatka crab IIC, Crayfish protease 1, Salmon enzyme 1, Atlantic cod I, or Atlantic cod II. A corneal ulcer associated with an infection is a particularly preferred target of this embodiment. In preferred embodiments, the composition used to treat a corneal wound also includes a microgel.

In yet another embodiment, the present invention provides a method for treating a surgical implant to reduce formation of adhesions following implantation of an implantable device comprising applying to the surface of the implantable device and to the area affected by the implantation an effective

amount of a composition comprising a microgel comprising a crosslinked polyanionic polymer that is made by polymerization of one or more ethylenically unsaturated crosslinking agents and one or more ethylenically unsaturated compounds at least one of which has:

i) one or more functional groups that can be titrated with base to form negatively charged functional groups. or

ii) one or more precursor groups that are precursors of the functional groups that can be titrated with base; which precursor groups are converted to the functional groups. In some embodiments, the crosslinked polyanionic polymer is made from a combination of one or more ethylenically unsaturated crosslinking agents and one or more ethyleneically unsaturated compounds that can be represented by the structure

$$(R^3)(R^2)C=C(R^1)\text{-}X\text{-}Y$$

wherein:

Y is $-C(O)OR^4$; $-S(O_2)OR^4$; or $-S(O)OR^4$; wherein R^4 is hydrogen, C_1 to C_6 alkyl, phenyl, or benzyl.

X is a direct bond; a straight or branched alkylene group having two to six carbon atoms, one or more of which can be replaced by O, S, or N heteroatoms, provided that there is no heteroatom in a position α or β to Y; phenylene; a five or six membered heteroarylene having up to three heteroatoms independently selected from O, S, and N, provided that neither Y or $R^3R^2C=C(R^1)$- is bonded to a heteroatom; and

R^1, R^2, and R^3 are independently selected from, hydrogen, C_1-C_6 alkyl, carboxy, halogen, cyano, isocyanato, C_1-C_6 hydroxyalkyl, alkoxyalkyl having 2 to 12 carbon atoms, C_1-C_6 haloalkyl, C_1-C_6 cyanoalkyl, C_3-C_6 cycloalkyl, C_1-C_6 carboxyalkyl, aryl, hydroxyaryl, haloaryl, cyanoaryl, C_1-C_6 alkoxyaryl, carboxyaryl, nitroaryl, or; and a group -X-Y; wherein C_1-C_6 alkyl or C_1-C_6 alkoxy groups are either linear or branched and up to Q-2 carbon atoms of any C_3-C_6 cycloalkyl group, wherein Q is the total number of ring carbon atoms in the cycloalkyl group, are independently replaced with O, S, or N heteroatoms; with the proviso that neither doubly-bonded carbon atom is directly bonded to O or S and wherein aryl is phenyl or a 5 or 6 membered heteroaryl group having up to three heteroatoms selected from the group consisting of O, S, and N. The hydrogel used in the method of treating an implant can include a protease.

Preferably, the microgel used in the method of treating an implantable device comprises a crosslinked polyanionic polymer made from a combination containing ethylenically unsaturated crosslinking agent in which the mole fraction of ethylenic double bonds in the combination that are attributable to the ethylenically unsaturated crosslinking agent is 0.02 or less. Preferably, the ratio of the macroviscosity of the microgel to the macroviscosity of the microgel is 10,000 or less.

In yet another embodiment, the invention provides a method for isolating a multifunctional enzyme from a biological specimen using fresh water without mechanically disrupting the biological specimen. In yet another embodiment, the method of isolation of multifunctional enzyme includes

applying a fresh water biological extract to an affinity column having a ligand, preferably aminophenylboronate.

In other embodiments, the present invention provides for the use of a microgel comprising a crosslinked polyanionic polymer in the manufacture of compositions that are useful for treatment of trauma, including corneal wounds and internal trauma. Corneal wounds include corneal ulcers, which can be infected, corneal abrasions, or chemical or physical insult to the cornea. Surgical insult is but one example of a physical insult. Trauma can be internal trauma that includes trauma to a membrane covering an internal organ or tissue, or covering a cavity in which one or more organs or tissues reside. Examples of membranes include the peritoneum, the epicardium, the pericardium, and the pleura. Other examples of membranes are epithelia, which includes endothelia, and the meninges. Internal trauma also includes trauma to a tendon or tendon sheath, or a nerve or nerve sheath.

In yet other embodiments, the present invention provides for use of microgels in the manufacture of compositions, which can also include proteins, in particular hyrolases, to be used to prevent the formation or reformation of adhesions. When a protein is used, it is preferably a protease, more preferably a protease, having and activity comprising at least two of a chymotrypsin, trypsin, collagenase, and elastase activity. A particularly preferred protein is a multifunctional enzyme that is (a) a first enzyme and has at least about 60% sequence similarity with a reference sequence which is AA64-300 of SEQ ID NO:2 or AA1-300 of SEQ ID NO:2 or a sequence differing from these by at least one of the residue differences found in SEQ ID NO:4, 6, 8, 10, or 12 or (b) a second enzyme which is *Panaeus vanameii* 1, *Panaeus vanameii* 2, *Panaeus monodon* chymotryptic-1, *Panaeus monodon* tryptic, *Panaeus monodon* chymotryptic-2, *Uca pugilator* enzyme I, *Uca pugilator* enzyme II, Kamchatka crab IA, Kamchatka crab IIA, Kamchatka crab IIB, Kamchatka crab IIC, Crayfish protease 1, Salmon enzyme 1, Atlantic cod I, or Atlantic cod II. The same microgels and proteins can be used in the manufacture of compositions that can be used to treat corneal wounds, especially corneal ulcers, that can be infected.

Preferred microgel that can be used in the manufacture of compositions useful for treating corneal wounds and internal trauma comprises crosslinked polyanionic polymers made by polymerization of one or more ethylenically unsaturated crosslinking agents and one or more ethylenically unsaturated compounds at least one of which has:

i) one or more functional groups that can be titrated with base to form negatively charged functional groups, or

ii) one or more precursor groups that are precursors of the functional groups that can be titrated with base; which precursor groups are converted to the functional groups. Preferred functional groups are selected from $-C(O)OR^4$; $-S(O_2)OR^4$; or $-S(O)OR^4$; wherein R^4 is hydrogen Preferred precursor groups are selected from $-C(O)R^4$, $-S(O_2)OR^4$, or $-S(O)OR^4$; wherein R^4 is independently C_1 - C_6 normal or branched alkyl, phenyl, or benzyl.

In particularly preferred embodiments, the microgel uses to manufacture a composition, that can contain a protein, for treating corneal wounds and internal trauma comprises a crosslinked polyanionic

polymer made from polymerization of a combination of at least one ethylenically unsaturated crosslinking agent and one or more ethylenically unsaturated compounds, at least one of which can be represented by the structure

$$(R^3)(R^2)C=C(R^1)\text{-}X\text{-}Y$$

wherein:

Y is $-C(O)OR^4$; $-S(O_2)OR^4$; or $-S(O)OR^4$; wherein R^4 is hydrogen, C_1 to C_6 normal or branches alkyl, phenyl, or benzyl.

X is a direct bond; a straight or branched alkylene group having two to six carbon atoms, one or more of which can be replaced by O, S, or N heteroatoms, provided that there is no heteroatom in a position α or β to Y; phenylene; a five or six membered heteroarylene having up to three heteroatoms independently selected from O, S, and N, provided that neither Y or $R^3R^2C=C(R^1)$- is bonded to a heteroatom; and

R^1, R^2, and R^3 are independently selected from, hydrogen, C_1-C_6 alkyl, carboxy, halogen, cyano, isocyanato, C_1-C_6 hydroxyalkyl, alkoxyalkyl having 2 to 12 carbon atoms, C_1-C_6 haloalkyl, C_1-C_6 cyanoalkyl, C_3-C_6 cycloalkyl, C_1-C_6 carboxyalkyl, aryl, hydroxyaryl, haloaryl, cyanoaryl, C_1-C_6 alkoxyaryl, carboxyaryl, nitroaryl; and a group -X-Y; wherein C_1-C_6 alkyl or C_1-C_6 alkoxy groups are either linear or branched and up to Q-2 carbon atoms of any C_3-C_6 cycloalkyl group, wherein Q is the total number of ring carbon atoms in the cycloalkyl group, are independently replaced with O, S, or N heteroatoms; with the proviso that neither doubly-bonded carbon atom is directly bonded to O or S; and wherein aryl is phenyl or a 5 or 6 membered heteroaryl group having up to three heteroatoms selected from the group consisting of O, S, and N. In more preferred embodiments, R^1, R^2, and R^3 are independently hydrogen or C_1 - C_3 alkyl and X is a direct bond. In particularly preferred embodiments, R^2 and R^3 are hydrogen, R^1 is either hydrogen or methyl, preferably methyl, X is a direct bond, and Y is COOH. In particularly preferred embodiments, the mole fraction of ethylenic double bonds in the combination, from which the crosslinked polyanionic polymer of the microgel used in manufacture is made, that is contributed by the ethylenically unsaturated crosslinking agent is 0.02 or less. Preferably the mole fraction of ethylenic double bonds in the mixture from which the crosslinked polyanionic polymer is made that is contributed by the ethylenically unsaturated crosslinking agent is 0.01 or less. The trially ether of sucrose and the triallyl ether of pentaerythritol are suitable crosslinking agents.

In yet another embodiment, the present invention provides for use of a microgel comprising a crosslinked polyanionic polymer in the manufacture of composition, that can contain a protein, especially a hydrolase, for preventionor minimization of adhesions. Preferably, the microgel comprises a polyanionic polymer made by polymerization of a combination of one or more ethylenically unsaturated crosslinking agents and one or more ethylenically unsaturated compounds at least one of which has:

i) one or more functional groups that can be titrated with base to form negatively charged functional groups, or

ii) one or more precursor groups that are precursors of the functional groups that can be titrated with base; which precursor groups are converted to the functional groups; and wherein at least one of the following is true:

iii) the mole fraction of total ethylenic double bonds in the combination from which the crosslinked polyanionic polymer is made that is contributed by the ethylenically unsaturated crosslinking agent is 0.02 or less, or

iv) the ratio of macroviscosity of the microgel to the microviscosity of the microgel is 10,000 or less.

In the forgoing embodiment, the functional groups are selected from $-C(O)OR^4$; $-S(O_2)OR^4$; or $-S(O)OR^4$; wherein R^4 is hydrogen; and precursor groups are selected from $-C(O)R^4$, $-S(O_2)OR^4$, or $-S(O)OR^4$; wherein R^4 is independently C_1 - C_6 normal or branched alkyl, phenyl, or benzyl. Preferably, at least one of the ethylenically unsaturated compounds in the combination has a structure that can be repesented by the structure

$$(R^3)(R^2)C=C(R^1)\text{-}X\text{-}Y$$

wherein:

Y is $-C(O)OR^4$; $-S(O_2)OR^4$; or $-S(O)OR^4$; wherein R^4 is hydrogen, C_1-C_6 alkyl, phenyl, or benzyl;

X is a direct bond; a straight or branched alkylene group having two to six carbon atoms, one or more of which can be replaced by O, S, or N heteroatoms, provided that there is no heteroatom in a position α or β to Y; phenylene; a five or six membered heteroarylene having up to three heteroatoms independently selected from O, S, and N, provided that neither Y or $R^3R^2C=C(R^1)$- is bonded to a heteroatom; and

R^1, R^2, and R^3 are independently selected from, hydrogen, C_1-C_6 alkyl, carboxy, halogen, cyano, isocyanato, C_1-C_6 hydroxyalkyl, alkoxyalkyl having 2 to 12 carbon atoms, C_1-C_6 haloalkyl, C_1-C_6 cyanoalkyl, C_3-C_6 cycloalkyl, C_1-C_6 carboxyalkyl, aryl, hydroxyaryl, haloaryl, cyanoaryl, C_1-C_6 alkoxyaryl, carboxyaryl, nitroaryl, or and a group -X-Y; wherein C_1-C_6 alkyl or C_1-C_6 alkoxy groups are either linear or branched and up to Q-2 carbon atoms of any C_3-C_6 cycloalkyl group, wherein Q is the total number of ring carbon atoms in the cycloalkyl group, are independently replaced with O, S, or N heteroatoms; with the proviso that neither doubly-bonded carbon atom is directly bonded to O or S and wherein aryl is phenyl or a 5 or 6 membered heteroaryl group having up to three heteroatoms selected from the group consisting of O, S, and N. Preferably, R^1, R^2, and R^3 are independently hydrogen or C_1-C_3 alkyl, X is a direct bond, and Y is $-C(0)OR^4$. More preferably, R^1, R^2, and R^3 are hydrogen and R^4 is hydrogen.

In yet other embodiments, the present invention provides for use of a protein in the manufacture of a composition for treating a corneal wound. Preferably, the protein is a multifunctional enzyme that is (a) a first enzyme and has at least about 60% sequence similarity with a reference sequence which is AA64-300 of SEQ ID NO:2 or AA1-300 of SEQ ID NO:2 or a sequence differing from these by at least

one of the residue differences found in SEQ ID NO:4, 6, 8, 10, or 12 or (b) a second enzyme which is *Panaeus vanameii* 1, *Panaeus vanameii* 2, *Panaeus monodon* chymotryptic-1, *Panaeus monodon* tryptic, *Panaeus monodon* chymotryptic-2, *Uca pugilator* enzyme I, *Uca pugilator* enzyme II, Kamchatka crab IA, Kamchatka crab IIA, Kamchatka crab IIB, Kamchatka crab IIC, Crayfish protease 1, Salmon enzyme 1, Atlantic cod I, or Atlantic cod II.

In yet another embodiment, the invention provides for use of a microgel in the manufacture of a composition, that can contain a protein, for prevention or reduction of adhesions following surgical implantation of an implantable device. The microgel can be made by polymerization of one or more ethylenically unsaturated crosslinking agents and one or more ethylenically unsaturated compounds at least one of which has:

i) one or more functional groups that can be titrated with base to form negatively charged functional groups, or

ii) one or more precursor groups that are precursors of the functional groups that can be titrated with base; which precursor groups are converted to the functional groups. Preferred functional groups are selected from $-C(O)OR^4$; $-S(O_2)OR^4$; or $-S(O)OR^4$; wherein R^4 is hydrogen Preferred precursor groups are selected from $-C(O)R^4$, $-S(O_2)OR^4$, or $-S(O)OR^4$; wherein R^4 is independently C_1 - C_6 normal or branched alkyl, phenyl, or benzyl.

In particularly preferred embodiments, the microgel uses to manufacture a composition, that can contain a protein, for preventing or minimizing adhesions following surgical implantation of an implantable device comprises a crosslinked polyanionic polymer made from polymerization of a combination of at least one ethylenically unsaturated crosslinking agent and one or more ethylenically unsaturated compounds, at least one of which can be represented by the structure

$$(R^3)(R^2)C=C(R^1)\text{-}X\text{-}Y$$

wherein:

Y is $-C(O)OR^4$; $-S(O_2)OR^4$; or $-S(O)OR^4$; wherein R^4 is hydrogen, C_1 to C_6 normal or branches alkyl, phenyl, or benzyl,

X is a direct bond; a straight or branched alkylene group having two to six carbon atoms, one or more of which can be replaced by O, S, or N heteroatoms, provided that there is no heteroatom in a position α or β to Y; phenylene; a five or six membered heteroarylene having up to three heteroatoms independently selected from O, S, and N, provided that neither Y or $R^3R^2C=C(R^1)$- is bonded to a heteroatom; and

R^1, R^2, and R^3 are independently selected from, hydrogen, C_1-C_6 alkyl, carboxy, halogen, cyano, isocyanato, C_1-C_6 hydroxyalkyl, alkoxyalkyl having 2 to 12 carbon atoms, C_1-C_6 haloalkyl, C_1-C_6 cyanoalkyl, C_3-C_6 cycloalkyl, C_1-C_6 carboxyalkyl, aryl, hydroxyaryl, haloaryl, cyanoaryl, C_1-C_6 alkoxyaryl, carboxyaryl, nitroaryl; and a group -X-Y; wherein C_1-C_6 alkyl or C_1-C_6 alkoxy groups are either linear or branched and up to Q-2 carbon atoms of any C_3-C_6 cycloalkyl group, wherein Q is the total number of ring carbon atoms in the cycloalkyl group, are independently replaced with O, S, or N

heteroatoms; with the proviso that neither doubly-bonded carbon atom is directly bonded to O or S; and wherein aryl is phenyl or a 5 or 6 membered heteroaryl group having up to three heteroatoms selected from the group consisting of O, S, and N. In more preferred embodiments. R^1, R^2, and R^3 are independently hydrogen or C_1 - C_3 alkyl and X is a direct bond. In particularly preferred embodiments. R^2 and R^3 are hydrogen, R^1 is either hydrogen or methyl, preferably methyl, X is a direct bond, and Y is COOH. In particularly preferred embodiments, the mole fraction of ethylenic double bonds in the combination, from which the crosslinked polyanionic polymer of the microgel used in manufacture is made, that is contributed by the ethylenically unsaturated crosslinking agent is 0.02 or less. Preferably the mole fraction of ethylenic double bonds in the mixture from which the crosslinked polyanionic polymer is made that is contributed by the ethylenically unsaturated crosslinking agent is 0.01 or less. The trially ether of sucrose and the triallyl ether of pentaerythritol are suitable crosslinking agents.

These and other aspects of the present invention are discussed hereinbelow.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the DNA sequence of a first isoform ("p62") (SEQ ID NO:1) of a krill-derived multifunctional protein aligned with the DNA sequence of a second isoform ("p31") (SEQ ID NO:3).

Figure 2 shows the amino acid sequence of the first isoform ("p62") (SEQ ID NO:2) of a krill-derived multifunctional protein aligned with the amino acid sequence of the second isoform ("p31") (SEQ ID NO:4).

Figure 3 shows the DNA sequence of a third isoform ("p5.1a") (SEQ ID NO:5) of a krill-derived multifunctional protein aligned with the DNA sequence of the first isoform ("p62") (SEQ ID NO:1).

Figure 4 shows the amino acid sequence of the third isoform ("p5.1a") (SEQ ID NO:6) aligned with the amino acid sequence of the first isoform ("p62") (SEQ ID NO:4) and the amino acid sequence of the second isoform ("p31") (SEQ ID NO:4).

Figure 5 shows the amino acid sequences of several proteins, namely, Factor VII, thrombin, kallikrein, a *Limulus* pro-clotting enzyme, plasmin, hepsin and Factor XII, aligned with the amino acid sequence of the first isoform ("p62") (SEQ ID NO:1).

Figure 6 shows sequence comparisons between the nucleic acid sequences for p62 (SEQ ID NO:1), p13 (SEQ ID NO:7), p912 (SEQ ID NO:9), p5.1b (SEQ ID NO:11) and p31 (SEQ ID NO:3), as well as sequence comparisons for the aligned peptide sequences (SEQ ID NOs:2, 8, 10, 12 and 4, respectively). Nucleic acid sequence differences in the open reading frames relative to p62 are indicated by underlining, and differences in amino acid sequence are indicated with recitals of the differing residues.

Figure 7 shows sequence comparisons between the polypeptide sequences encoded by p62 (SEQ ID NO:2), p13 (SEQ ID NO:8), p912 (SEQ ID NO:10), p5.1b (SEQ ID NO:12) and p31 (SEQ ID NO:4).

Figure 8 illustrates a sequence alignment between the polypeptide sequences encoded by p62 (SEQ ID NO:2), p912 (SEQ ID NO:10), p5.1a (SEQ ID NO:6) and p31 (SEQ ID NO:4).

DETAILED DESCRIPTION

For the purposes of this application, the terms listed below shall have the following respective meanings:

• **acid number** refers to the amount of potassium hydroxide in milligrams needed to neutralize a gram of a dry material. A material is dry if it contains not more that 2% by weight of water, an organic solvent, or organic monomer.

• **enzymatically active segment** means a segment of a multifunctional protein having activity comprising at least one of a chymotrypsin, trypsin, collagenase, elastase or exo peptidase activity.

• **effective amount:** The meaning of "effective amount" will be recognized by clinicians but includes an amount effective to (1) reduce, ameliorate or eliminate one or more symptoms of the disease sought to be treated or the condition sought to be avoided, (2) induce a pharmacological change relevant to treating the disease sought to be treated or condition sought to be avoided, (3) prevent or lessen the frequency of occurrence of a disease or condition, (4) inhibit or prevent infection or re-infection by an infective agent, (5) prevent the occurrence of a non-infectious disease (for instance a disease treatable by blocking a cell adhesion phenomenon), (6) increase the rate of healing of a wound (e.g., average time it takes for a wound to heal), (7) decrease the amount of scarring resulting from wound healing, or (8) inhibit or prevent formation or reformation of adhesions.

• **hydrogel** is a combination with water of a hydrophilic polymer, which may be linear, branched, covalently crosslinked, ionically crosslinked, physically crosslinked, or crosslinked by hydrogen bonding. A hydrogel has 50% or more water by weight. Examples of hydrophilic polymers that form hydrogels are carboxymethylcellulose and carboxypolymethylene.

• **hydrolase** means an enzyme that degrades bonds formed by dehydration reactions such as amide, ester, or ether bonds. The term encompasses, but is not limited to, proteases such as trypsin and chymotrypsin.

• **identity**, as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, or portions thereof, as determined by comparing the sequences or strings of such sequences. Whereas "identity" relates to the comparison of the individual subunits of a polynucleotide or a polypeptide, the related term "similarity" relates to differences in the respective subunits of a polypeptide only where a difference of identity in a particular position can result in greater or lesser similarity due to whether the differing amino acid subunit has similar or different chemical characteristics, as are known in the art. One can readily calculate percentage identity or similarity by known methods, including but not limited to those described in *Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data*, Part 1, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM *J. Applied Math., 48:* 1073 (1988).

Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include. but are not limited to, the GCG program package (Devereux, J., et al., *Nucleic Acids Research 12(1):* 387 (1984)), BLASTP, BLASTN, and FASTA (Atschul, S.F. et al., *J. Molec. Biol. 215:* 403-410 (1990)). The BLAST X program is publicly available from NCBI and other sources (*BLAST Manual*, Altschul, S., *et al.*, NCBI NLM NIH Bethesda, MD 20894; Altschul, S., *et al., J. Mol. Biol. 215*: 403-410 (1990)). The well known Smith Waterman algorithm may also be used to determine identity.

Preferred parameters for polypeptide sequence comparison include the following: (1) Algorithm: Needleman and Wunsch, J. Mol. Biol. 48: 443-453 (1970); (2) Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, Proc. Natl. Acad. Sci. USA. 89:10915-10919 (1992); (3) Gap Penalty: 12; and (4) Gap Length Penalty: 4. A program useful with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison WI. The aforementioned parameters are the default parameters for peptide comparisons (along with no penalty for end gaps).

Preferred parameters for polynucleotide comparison include the following: (1) Algorithm: Needleman and Wunsch, J. Mol Biol. 48: 443-453 (1970); (2) Comparison matrix: matches = +10, mismatch = 0; (3) Gap Penalty: 50; and (4) Gap Length Penalty: 3. A program useful with these parameters is publicly available as the "gap" program from Genetics Computer Group, Madison WI. The aforementioned parameters are the default parameters for nucleic acid comparisons.

Preferred polypeptide embodiments further include an isolated polypeptide comprising a polypeptide having at least a 60, 70, 80, 85, 90, 95, 97 or 100% identity to a polypeptide reference sequence such as SEQ ID NO:2, or a portion thereof, more particularly AA64-300 thereof, wherein the sequence of the polypeptide can be identical to the reference sequence or can include up to a certain integer number of amino acid alterations as compared to the reference sequence. The alterations are selected from the group including at least one amino acid deletion, substitution, including conservative and non-conservative substitution, or insertion, and wherein said alterations may occur at the amino- or carboxy-terminal positions of the reference polypeptide sequence or anywhere between those terminal positions, interspersed either individually among the amino acids in the reference sequence or in one or more contiguous groups within the reference sequence. The number of amino acid alterations is determined by multiplying the total number of amino acids in the reference sequence by the numerical percent of the respective percent identity and subtracting that product from said total number of amino acids in the reference sequence, or:

$$n_a \leq x_a - (x_a \bullet y),$$

wherein n_a is the number of amino acid alterations, x_a is the total number of amino acids in the reference sequence, or a portion thereof, and y is 0.60 for 60% identity, 0.70 for 70%, 0.80 for 80%, 0.85 for 85%, 0.90 for 90%, 0.95 for 95%, 0.97 for 97% or 1.00 for 100%, and wherein any non-integer product of x_a

and y is rounded down to the nearest integer prior to subtracting it from x_a. Restated, relatedness between two strings of sequence (a query string and a reference string) can be stated in terms of a query sequence that is identical with the reference sequence, or, if not identical, then over the entire length corresponding to the reference sequence, the nucleic acid sequence has an average of up to thirty (or twenty, ten, five, two or one) substitutions, deletions or insertions for every 100 nucleotides or amino acid residues of the reference sequence.

• **isoform** means a naturally occurring sequence variant of a substantially homologous protein within the same organism. Preferably, the isoform shares at least about 80% identity, and more preferably, at least about 85% identity with a reference sequence.

• **krill-derived multifunctional protein** means a multifunctional protein having the same sequence as a protein isolated from krill having the properties of the protein described in the section entitled "Preferred Characteristics of the Multifunctional Protein." This protein is also referred to as the "krill-derived multifunctional hydrolase" and includes all isoforms of the protein. The amino acid sequence included in SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10 or SEQ ID NO:12 or other isoforms thereof or chimeric polypeptides thereof are examples of krill-derived multifunctional proteins.

• **microgel** means a viscoelastic mass of discrete particles, each discrete particle comprising crosslinked polyanionic polymer and each particle having a size in its aqueous swollen state at neutral pH of between 0.1 and 1000 μm. The particles of aqueous swollen polyanionic polymer have 70% or more water and the crosslinking is ionic, covalent, or through hydrogen bonding.

• **microviscosity** is measured, for example, by any method set forth in R.Y. Lochhead et al., "Poly(acrylic acid) Thickeners: The Importance of Gel Microrheology and Evaluation of Hydrophobically Modified Derivatives as Emulsifiers," in *Polymers in Aqueous Media*, pp. 113-147, 1989, which document is incorporated by reference herein in its entirety. One such method measures microdiffusion with bimodal gold sols, for example allowing for microdiffusion to be measured for a microstructure centered around 10 nm and 100nm

• **multifunctional protein** means a protein having activity comprising at least one of a chymotrypsin, trypsin, collagenase, elastase or exo peptidase activity or asialo GM_1 ceramide binding activity, and substantial homology to at least a segment of a krill-derived multifunctional protein.

• **neutral functional group** means a functional group that is not titrated by acid or base.

• **nucleic acid** means the nucleic acid sequence embodiments of the invention are preferably deoxyribonucleic acid sequences, preferably double-stranded deoxyribonucleic acid sequences. However, they can also be ribonucleic acid sequences, or nucleic acid mimics, meaning compounds designed to preserve the hydrogen bonding and base-pairing properties of nucleic acid, but which differ from natural nucleic acid in, for example, susceptibility to nucleases.

• **physiological pH** means a pH between 6.5 and 7.5.

• **polyanionic polymer** means a polymer having an acyclic backbone and having ionizable functional groups , for example carboxy groups, that become negatively charged functional groups, for example

carboxylate anions, at physiological pH. A gram of polyanionic polymer has 0.001 moles or more of functional groups that can be titrated with KOH. The ionizable functional groups can be directly chemically bonded to the polymer backbone or they can be chemically bonded to a side group or side chain that is in turn chemically bonded to the main chain. Carboxypolymethylene is an example of a polyanionic polymer in which the ionizable functional group is directly bonded to the main chain.

• **reference protein or sequence** means a reference protein sequence which is AA64-300 of SEQ ID NO:2 or AA1-300 of SEQ ID NO:2 or a sequence differing therefrom by at least one of the residue differences or extensions found in SEQ ID NOs:4, 6, 8, 10, or 12. A reference protein is a protein having the reference protein sequence. With reference to Figure 6, examples of reference proteins are (a) a protein with sequence of AA64-300 of SEQ ID NO:2 except that residue 128 is serine or (b) a protein with sequence of SEQ ID NO:2 except that before Leu^1 is serine. In a preferred embodiment, the N-terminal has additional sequence from SEQ ID NOs:10 and 12 such as NH_2-Ala, NH_2-IleAla, NH_2-ArgIleAla, NH_2-SerArgIleAla (SEQ ID NO:44), NH_2-ArgSerArgIleAla (SEQ ID NO:45), NH_2-GlyArgSerArgIleAla (SEQ ID NO:45) or NH_2-ProGlyArgSerArgIleAla (SEQ ID NO:46). Reference nucleic acid sequences are the corresponding coding regions of SEQ ID NO:1, or a sequence differing therefrom by one of the nucleotide differences or extensions found in SEQ ID NOs:1, 5, 7, 9 or 11.

• **substantial homology** means at least about 60% sequence identity or similarity, for example 60% sequence identity.

• **unit of multifunctional hydrolase** ("U"), as used herein with respect to the krill broad specificity serine protease and related such enzymes, is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of substrate per minute at 25°C, wherein succinyl-ala-ala-pro-phe-*p*-nitroanilide (Sigma Chemical Co., St. Louis, MO) is the substrate, and hydrolysis is monitored via the absorbance change at 410 nm. The extinction coefficient ε of *p*-nitroanilide is 8800 $M^{-1}cm^{-1}$, thus the multiplication factor to convert dA/minute into U/minute of sample is 5.68, when 20 μl of sample is used.

The present invention provides a method for treating wounds and other trauma to anatomical membranes of a metazoan, including but not limited to mammals, humans, food animals, such as cows, pigs, sheep, goats, and the like, companion animals, such as dogs, domestic cats, horses, and the like, and exotic animals, such as elephants, apes, large cats, whales, and the like. The term membrane is used broadly and includes tissue boundaries and tissue surfaces, such as the dura mater and the surfaces of tendons; the anterior limiting area of the cornea; membranes covering internal organs or lining the cavities in which the organs reside, which would include tendons within their sheaths; and internal and external epithelia and mesothelia. The term epithelium is herein used in its broadest sense and will be understood to refer to simple, stratified, and transitional epithelia, as well as the endothelium of serous membranes. The epidermis and the conjunctival epithelium on the *substantia propria* of the cornea are external epithelia. Internal epithelium includes surfaces, which are sometimes denoted endothelia, such as the peritoneum, pleura, and pericardium and like membranes that cover internal structures and organs, such as the viscera, the body cavity wall, and the like.

The term trauma is intended to encompass any wound, insult, or noxious stimulus to a membrane or tissue surface. Trauma that is treated by the method of the present invention may or may not result in breach of the membrane or tissue boundary. Wounds can result from a disease condition, for example vascular insufficiency or infection associated with a pathogen, burns (thermal or chemical), or from application of external force to a membrane or tissue surface by accident or surgery. Noxious stimuli includes the action of heat or corrosive chemicals, for example acids and caustics, as well as manipulation of an organ during surgery.

The term corneal wounds is intended to encompass any injury to the cornea, for example, infection by a pathogen, a corneal abrasion, a corneal ulcer, or an insult capable of giving rise to a corneal ulcer in a mammal, including but not limited to humans, food animals, such as cows, pigs, sheep, goats, and the like, companion animals, such as dogs, domestic cats, horses, and the like, and exotic animals, such as elephants, apes, large cats, whales, and the like. An insult capable of giving rise to a corneal ulcer can be chemical, for example exposure to a corrosive chemical, or it can be physical, for example impact by a foreign object or a surgical incision as in keratoplasty or keratotomy.

The method of the present invention is particularly useful in the treatment of, for example, ulcerations and other injuries of corneal tissue as well as cutaneous wounds such as decubitus ulcers, venous ulcers, burns, or pressure sores. Treatment of corneal ulcers according to the method of the present invention retards or arrests growth of the ulcer, which, if left unchecked, can lead to perforation. Treatment of corneal ulcers according to the method of the present invention also improves the rate of healing, that is the rate of return of the cornea or skin to its pre-trauma condition, reducing the risk of opportunistic infection, and prevents or reduces formation of scar tissue. A preferred target of the present inventive method of treatment is a corneal ulcer that is associated with an infection, such as viral infection caused by a Herpes virus (HSV), or a bacterial infection, such as one of a pseudomonad or a *Moraxella* species, as in *Moraxella bovis* that causes corneal ulcerations in cattle. In one embodiment, the present method comprises administering to an affected area of the cornea an effective amount of a composition containing a microgel, for example a microgel from a crosslinked carboxypolymethylene. Treatment of cutaneous wounds by the present method preferably includes application to an area affected by a cutaneous wound of a microgel for which the ratio of macroviscosity to microviscosity is 10,000 or less and that, in yet more preferred embodiments, contains a multifunctional krill-derived hydrolase. The treating practitioner will understand that area affected by a cutaneous wound varies with the size, location, and severity of the wound but includes the wound itself and an area cm around the wound (such as with 3 cm).

The method of the present invention is likewise particularly useful in the prevention or reduction of the incidence or severity of adhesions, for example those that frequently form between the peritoneum and viscera, or between non-adjacent areas of the peritoneum, following surgical procedures that inflict internal trauma, including internal surgical wounds. Adhesions are scar tissue that first develops as fibrous bands between two tissue surfaces that, despite being in apposition, normally have free movement

relative to each other. The adhesions arise as a result of repair processes after an insult or a noxious stimulus has damaged the integrity of one or both opposing surfaces. Noxious stimuli include trauma (both surgical and accidental), infection, and any physical or chemical agent that can cause inflammation leading to a repair response. When adhesions prevent the normal movement between the affected surfaces, dysfunction of the underlying organ or pain may result. Adhesions start as thin and filmy strands, largely composed of fibrin, which are easily disrupted at this stage. With time they become organized, laying down collagen and becoming vascularised. At this stage, only surgical division will separate the adhering structures. This becomes necessary when the function of the tethered organ is impaired or viability is at risk. The method, as it relates to prevention of post-operative formation of adhesions, is applicable to other types of adhesions apart from those of the peritoneum. For example, prevention of formation or reformation of adhesions after adhesiolysis, tendon surgery, thoracic surgery, abdominal surgery, eye or ear surgery, spinal surgery, nerve surgery, pelvic surgery, gynecological surgery, as well as after surgery on the cranium, brain, and spinal cord.

The present method comprises treating the affected area of, in, or around a trauma; for example a surgical incision, or corneal ulcer or injury by applying to the affected area a trauma-treating effective amount of a composition that includes either a microgel, a multifunctional hydrolase or both. The hydrolase can be a protease, particularly a multifunctional krill-derived serine protease. In a preferred embodiment, the present method comprises treating the area affected by a corneal wound or surgical wound with a composition that includes a microgel that can contain a protease, preferably a multi-functional krill-derived protease.

The skilled artesian will recognize that the area affected by trauma of any type (the affected area) will depend on the nature, size, and location of the trauma. By way of example, in the case of a corneal wound, the affected area can be the entire exposed surface of the eye. When the trauma is an internal surgical wound involving a body cavity, the affected area includes surfaces of organs or tissues in the body cavity into which the surgical incision (wound) is made. In the case of peritoneotomy, the affected area is the entire peritoneal cavity and the organs residing within the peritoneal cavity; in the case of thoracotomy, the affected area is the entire thoracic cavity and the organs residing within the thoracic cavity. In the case of tendon surgery, the affected area includes the area of the incision and extends from 1 or 2 to as many as 15 cm from the incision of the tendon sheath and includes the surfaces of tissues surrounding the tendon and its sheath.

In one embodiment, the present method comprises treating the area affected by an internal trauma to reduce post trauma formation of adhesions by applying to the affected area an effective amount of a microgel. The microgel includes a crosslinked polyanionic polymer. Typically, the amount of polyanionic polymer in the microgel is between 0.5 and 2.5 weight percent. In preferred embodiments, the microgel has 1% of crosslinked polyanionic polymer.

In one embodiment, the present method comprises treating the affected area of a cutaneous wound, such as a decubitus ulcer, venous ulcer, burn, or pressure sore, by applying to the affected area an

effective amount of a composition comprising a microgel that can contain a hydrolase, preferably a protease, most preferably a multifunctional krill-derived serine protease. An effective amount of a composition of this embodiment of the present invention is an amount sufficient to promote debridement and to prevent odor and unwanted seepage and infection in the cutaneous wound, and to cause it to heal faster that it would if it were merely cleansed and dressed.

In another embodiment, the present method comprises treating the affected area of the peritoneum, the epicardium, pericardium, or the pleura traumatized by an internal trauma by applying an effective amount of a composition comprising a microgel that can contain a krill-derived protease, preferably a protease, more preferably a multifunctional krill-derived protease.

In yet another embodiment, the present method comprises treating the area of the spine, the meninges, for example dura mater, or nerves and nerve sheaths traumatized by surgery or injury to reduce or prevent formation of adhesions by applying an effective amount of a composition comprising a microgel described above that can contain a protease , preferably a krill-derived protease. Alternatively, such affected area can be treated with a hydrolase, preferably a multi-functional krill-derived hydrolase, in any pharmaceutically acceptable vehicle carrier as is known in the art.

In still another embodiment, the present method comprises treating the area of a tendon and its sheath affected by internal trauma, for example a surgical wound as in tendoplasty.

In another embodiment, the present method comprises treating the area affected by internal trauma to reduce formation of adhesions by applying to the affected area an effective amount of a krill-derived multifunctional protease. The krill-derived multifunctional protease can be applied to the affected area in any pharmaceutically acceptable vehicle of the known art. Pharmaceutically acceptable vehicles serve as carriers for administration of pharmacologically active material such as the multifunctional protease of the present invention but do not interfere with the action of the active material or the bodily functions of the animal to which it is administered. Isotonic saline solution is an example of a pharmaceutically acceptable vehicle. Pharmaceutically acceptable vehicles can have excipients known in the art such as dextran, calcium chloride, glycine, citric acid, and sorbitol, to mention a few.

Compositions of the present invention containing crosslinked polyanionic polymers can also be applied to the area affected by bowel, thoracic, cranial, tendon, and gynecological surgery to prevent or reduce the formation or reformation of adhesions.

In yet another embodiment, the present invention provides a method for treating a surgical implant with a composition comprising a microgel to reduce adhesion formation between the implant and areas of tissue surrounding the implant or between different areas of the tissue surrounding the implant by applying to the surface of the surgical implant a coating including the composition having a thickness from between about 0.1 mm to about 5mm. Surgical implants with which the method can be used include joint and bone prostheses, including prosthetics of the inner ear, cranial plates, and cardiac pacemakers, drug delivery implants and in-dwelling catheters, among others.

The crosslinked polyanionic polymers that form the microgels used in the method of the present invention can be made by any method that provides a crosslinked polymer having an acyclic backbone and functional groups capable of ionizing to an anionic form under physiological conditions. For example, the polyanionic polymers used in the method of the present invention can be obtained by polymerization of a mixture that includes an ethylenically unsaturated crosslinking agent and at least one monomer that has an ionizable functional group capable of becoming negatively charged. Typically, the ionizable functional group is a base-titratable functional group. The carboxy group is an example of a base titratable functional group. The polyanionic polymer can also be obtained from a precursor polymer having precursor functional groups that can be hydrolyzed to the ionizable functional groups that, in turn, can become negatively charged. For example, a carboxylate ester is a precursor for a carboxy group which, when treated with base, becomes a negatively charged carboxylate anion. The precursor polymer can be obtained by polymerization of a mixture that includes one or more monomers at least one of which has a precursor for a functional group that is capable of becoming negatively charged. The precursor group can be converted to the functional group capable of becoming negatively charged by, for example, hydrolysis, or any other means as will be obvious to one skilled in the art from inspection of the chemical structure of the precursor group. Conversion of the precursor group can be made to occur prior to, at the time of, or after administration of a composition of the present invention.

The backbone, or main chain, of polyanionic polymers useful in the practice of the present invention includes repeat units that can be derived from polymerization of one or more monomers of structure I, wherein the double bond shown is disposed to polymerization at least by free radical polymerization.

$$(R^3)(R^2)C=C(R^1)\text{-}X\text{-}Y$$

I

In structure I, R^1, R^2, and R^3 can be independently selected from, hydrogen, alkyl having 1 to 6 carbon atoms (a C_1 - C_6 alkyl group), carboxy, halogen, cyano, isocyanato, hydroxyalkyl, alkoxyalkyl, haloalkyl, cyanoalkyl, cycloalkyl, carboxyalkyl, aryl, hydroxyaryl, haloaryl, cyanoaryl, carboxyaryl, or R^1, R^2, and R^3 can also be a group -X-Y, as these structures are defined below. The alkyl and alkoxy groups in the foregoing list may be linear or branched and preferably have from one to six carbon atoms. The cycloalkyl group preferably has five or six carbon atoms, one or more of which can be independently replaced with O, S, or N heteroatoms such that up to Q-2 carbon atoms of the cycloalkyl group (Q being the total number of carbon atoms in the cycloalkyl ring) can be replaced with heteroatoms.

In structure I, X is a direct bond or is a straight or branched alkylene group, preferably having two to six carbon atoms, one or more of which can be replaced with O, S, or N heteroatoms, provided that there is no heteroatom in a position α or β to Y. In structure I, X can also be phenylene, preferably 5 or 6 membered arylene having up to two heteroatoms independently selected from O, S, and N, with the

proviso that Y and $R^3R^2C=C(R^1)$- are not bonded to a heteroatom. Phenylene, oxazolylene, isoxazolylene, pyridazinylene, pyrimidinylene are examples of preferred arylenes.

In structure I, Y is $-C(O)OR^4$, $-S(O_2)R^4$, $-S(O)OR^4$; wherein R^4 is hydrogen, lower alkyl, especially C_1 to C_6 alkyl, phenyl, or benzyl. The group Y can be present in the monomer or the monomer can include a precursor group for Y, which is then formed in a post-polymerization reaction on polymer formed from monomer having the precursor group. By way of example, a polymer having methyl carboxylate groups, derived for example from methyl methacrylate, can be reacted with water and to produce a polymer having carboxy groups (Y = -COOH).

In the above structure, aryl means phenyl or a 5 or 6 membered heteroaryl group having up to Q-2 heteroatoms independently selected from O, S, and N; wherein Q is the total number of atoms in the ring.

Examples of suitable monomers include acrylic acid, methacrylic acid, allyl sulfonic acid, itaconic acid, maleic acid or its anhydride, itaconic acid, citraconic acid, to mention a few. Many other monomers that can be used to make polyanionic polymers that form microgels with water are described by Huang et al., United States Patent 4,509,949, incorporated herein by reference.

In reference to crosslinked polyanionic polymers that can form microgels, the term backbone and main chain are used interchangeably and will be understood to refer to that portion of the polymer chains not derived from crosslinking agents.

The polyanionic polymers used in the method of the present invention can be homopolymers, having repeat units derived from only one monomer described by structure I, or they can be multipolymers derived from polymerization of a mixture of any number of monomers of structure I. Co-, ter-, quatra-, and other multipolymers can include repeat units from monomers that do not bear ionizable groups or precursors therefor, for example styrene, that are capable of copolymerizing with the monomers of structure I, with the proviso that the final polymer has 0.001 or more moles, preferably 0.0014 or more moles, more preferably 0.01 mole or more, of base titratable functional groups per gram of polymer (on a commercially acceptable dry basis). A base titratable functional group is a functional group, for example a carboxy group, that can be titrated with KOH.

In preferred embodiments, polyanionic polymer is crosslinked and forms a microgel when combined with water. Preferred crosslinked polyanionic polymers are chemically crosslinked. Chemical crosslinking can be by ionic or covalent bonds, preferably it is by covalent bonds. The crosslinking can be introduced at the time the polyanionic polymer is made, or it can be introduced after the polyanionic polymer is made.

Preferably, crosslinking by covalent bonds is introduced at the time the polyanionic polymer is made by using one or more chemical crosslinking agents that have at least two ethyleneically unsaturated carbon-carbon double bonds disposed to polymerize by the same mechanism as the monomers represented by structure I, preferably a free radical mechanism. Examples of crosslinking agents that can be introduced at the time the polyanionic polymer is made include divinyl benzene and alkenyl ethers of

polyhydric alcohols, for example the triallyl ether of pentaerythritol available from Aldrich Chemical (catalog 25-172-0), among others. Commercially available ethylenically unsaturated ethers or esters of those polyhydric alcohols having 3 or more hydroxyl groups typically are provided as a mixture in which some of the hydroxyl groups may be underivatized. Reference herein to a particular degree of etherification or esterification, for example tri- or tetra-, will be understood to also refer to commercially important mixtures of etherified or esterified polyhydric alcohols as are known in the art to include minor amounts of etherified or esterified polyhydric alcohols having a lower or higher than indicated degree of etherification or esterification. Thus, reference to a particular mole fraction of double bonds will be understood to encompass the variation that would be expected because of this known variation in the degree of derivatization.

Preferably the amount of crosslinker is kept low. Preferred crosslinked polyanionic polymers form microgels with water and are made by polymerization of a mixture of one or more monomers of structure I and one or more ethylenically unsaturated crosslinking agents of the type discussed above. The amount of crosslinking agent or agents used is effective to produce a crosslinked polyanionic polymer that forms a microgel when combined with water. When ethylenically unsaturated crosslinking agents are used to form crosslinks at the time of making the polyanionic polymer, the ethylenic double bonds of the one or more ethylenically unsaturated crosslinking agents preferably account for less than 0.02 mole fraction and preferably less that 0.01 mole fraction of all ethylenically unsaturated double bonds in the combination of one or more monomers and one or more crosslinking agents. Typically, the ethyleneically unsaturated crosslinking agent accounts for 0.001 mole fraction or more of all ethylenic double bonds in the combination from which the crosslinked polyanionic polymer is made. These mole fractions are calculated on the basis of the nominal number of ethylenic double bonds in the ethylenically unsaturated crosslinking agent and are adjusted for the known variation in the average number of double bonds per molecule of commercially available ethylenically unsaturated crosslinking agents as discussed above.

In certain embodiments, the polyanionic polymer employed in the practice of the method of the present invention has an acid number of at least about 100, more preferably at least about 200, yet more preferably at least about 400, still yet more preferably at least about 600, still more preferably at least about 700 , when the polymer is in a commercially acceptable "dry" preparation such as a preparation containing the polymer and for example up to 2% moisture, residual solvent, or residual monomer. In preferred embodiments, the polyanionic polymer has 0.001 moles or more, preferably 0.0014 moles or more, more preferably 0.014 moles or more, of base titratable functional groups per gram of polymer in a commercially acceptable dry formulation.

The polyanionic polymers preferably have, in a 0.5% w/v neutralized aqueous solution (e.g. pH between 6 to 8), a Brookfield RVF or RVT viscosity, which is a measure of macroviscosity, of at least about 2,000 cP, more preferably at least about 4,000 cP (20 rpm at 25°C). These viscosity parameters are with respect to the acid form of the polymers. See, R.Y. Lochhead et al., *Polymers in Aqueous Media*, pp. 113-147, 1989 on macroviscosity (Brookfield viscosity) and microviscosity of polymer solutions.

However, in certain preferred embodiments, the macroviscosity is no more than about 100,000 times greater than the microviscosity, preferably no more than about 10,000 times greater.

In certain embodiments, microgels can have a particle size between 1 and 500 μm. In other embodiments the microgel can have a particle size between 10 and 500 μm.

In certain embodiments, the crosslinked polyanionic polymer is a crosslinked homopolymer or copolymer of acrylic acid, such as the polymers sold by the BFGoodrich Company, Specialty Polymers and Chemicals Division (Brecksville, OH) under the tradename Carbopol, such as carbopol 971P, Carbopol 934P and Carbopol 974P, which are preferred in the order: 971P more than 934P; and 934P more than 974P. These types of polymers have a substantially acyclic aliphatic backbone and have been termed carboxypolymethylenes or carbomers, which can be composed of any suitable number of monomers, and in a particular treatment, can be of a uniform number of such monomers or of a variable number of monomers per preparation applied to an area affected by a wound. Additionally, carboxypolymethylene can have a variable number of carboxyl groups attached to the polymethylene backbones. As crosslinker, the triallyl ether of pentaerythritol (at 0.1% to 2.5 % ,w/w, based on other monomers) is suitable.

Suitable salts can be combined with a microgel, the suitability of which is determined by the requirement that the microgel itself not cause harm to the injured cornea, peritoneum, or any other tissue with which the microgel comes in contact. Suitable salts include, but are not limited to, potassium or sodium chloride, particularly when provided at physiological concentrations, as are known in the art.

A composition used in the practice of the method of the present invention can include glycerol, the aforementioned carboxypolymethylene, and distilled water, and is adjusted as to pH using a base such as sodium hydroxide potassium hydroxide, alkyl amines such as diisopropanolamine (DIPA), and the like. A stock solution of a suitable concentration of glycerol can be prepared with distilled water, and is preferably an 87% (w/w) glycerol solution, the remainder of which is distilled water. A stock solution of a suitable solution of base such as sodium hydroxide can also be prepared with distilled water, for example, a 10% (w/w) sodium hydroxide solution, the remainder of which is water. By making appropriate dilutions of stock solutions, as is well known in the art, the microgel useful in the practice of the present method preferably has the following ranges of end concentrations of the aforementioned ingredients: (1) glycerol, from about 0 to about 60% (w/w); (2) carboxypolymethylene, from about 0.1% to about 10% (w/w), more preferably from about 0.4% to about 7%, yet more preferably, from about 1% to about 5%; the remainder of the formulation being distilled water. Sodium hydroxide, 10% stock, is used for pH adjustment, resulting in an essentially neutral prepared pH, more preferably a pH from about 7 to about 7.8, yet more preferably a pH from about 7.2 to about 7.6.

When inclusion of a hereinbelow described multifunctional hydrolase is contemplated, the microgel can also be prepared with excipients intended to protect the multifunctional hydrolase upon freeze drying or upon the reconstitution thereof with distilled water, or both. Such excipients include, for example, calcium chloride, glycine, citric acid, sorbitol, and dextran. A vial that contains, for example,

50 units of the aforementioned multifunctional hydrolase (which units are defined above) when freezedrying is contemplated, preferably includes the following excipients in the range of concentration given: (1) calcium chloride, from about 0.6 mM to about 1 mM, (2) glycine, from none up to about 12 mM, preferably from about 6 mM to about 10 mM, most preferably about 8 mM; (3) citric acid, from none up to about 12 mM, preferably from about 6 mM to about 10 mM, most preferably about 8 mM; (4) sorbitol, from about 100 mM to about 200 mM, preferably between about 150 mM and 170 mM, most preferably about 160 mM; and (5) dextran, from about 1% to about 10% by weight, preferably between about 7% to about 8% by weight, most preferably 6% by weight.

A preferred embodiment of the present invention provides for treatment of wounds, especially cutaneous wounds, with an above described microgel, optionally combined with a suitable multifunctional hydrolase. The multifunctional hydrolase preferably has proteolytic activity corresponding to that of at least one from the group comprising a chymotrypsin, trypsin, collagenase, elastase and exo peptidase activity. More preferably, the multifunctional hydrolase has at least two of said proteolytic activities; yet more preferably, at least three of said proteolytic activities; even more preferably, at least four of said proteolytic activities; and most preferably, all of said proteolytic activities.

The compositions used in the context of the method of the present invention are intended for topical application with respect to the area to be so treated. Alternatively, systemic and oral modes of treatment are contemplated as well. Microgels can be applied as paste, jelly, or in sheets that can be prehydrated or hydrated in situ by bodily fluids.

For topical administration to an area affected by internal trauma, for example an internal surgical wound that is susceptible to giving rise to adhesions, microgel can be administered as a paste, jelly, or pourable liquid formulation. For treatment of internal trauma, multifunctional krill-derived protein can be administered in a pharmaceutically acceptable vehicle, for example isotonic saline solution. The multifunctional protein can also be administered to an area affected by an internal surgical wound in a composition that includes a microgel. In preferred embodiments, the multifunctional protein is administered to the area affected by a surgical wound in a composition that contains a microgel. A particularly preferred microgel contains a crosslinked polyanionic polymer. Crosslinked carboxypolymethylene is a preferred crosslinked polyanionic polymer.

The method of treating trauma to a membrane, for example the peritoneum, pleura, or pericardium, or for treating trauma to an internal organ, comprises interoperatively administering a composition of the present invention to the site of the trauma and the affected area. Treatment to suppress formation or reformation of surgical adhesions is performed interoperatively.

Treatment of corneal wounds can be effected using hydrolase in any pharmaceutically acceptable vehicle according to standard pharmaceutical practice. The vehicle can be a microgel. Treatment is effected by application of drops or a gel to the eye. For ocular administration, ointments or droppable liquids may be delivered by ocular delivery systems known to the art such as applicators or eye droppers. Such compositions for treatment of corneal wounds can include mucomimetics such as hyaluronic acid.

chondroitin sulfate, hydroxypropyl methylcellulose or polyvinyl alcohol, preservatives such as sorbic acid, EDTA or benzylchronium chloride, and the usual quantities of diluents and/or carriers. In preferred embodiments, the composition containing multifunction protein to be administered to the eye includes a microgel, especially a microgel that includes a polyanionic polymer of the type described above.

The method of treating a corneal ulcer, such as one caused by a Herpes keratitis infection, for example, preferably comprises administering to an affected eye a composition comprising the aforementioned multifunctional hydrolase, wherein a corneal ulcer treating effective amount of the multifunctional hydrolase is administered, and wherein the multifunctional hydrolase preferably has at least two of a chymotrypsin, trypsin, collagenase, elastase or exo peptidase activity, and at least about 60% sequence similarity with a reference sequence. More preferably, the aforementioned hydrolase has at least three of said proteolytic activities and at least about 80% sequence identity with the reference sequence. Yet more preferably, the aforementioned hydrolase has at least three of said proteolytic activities and at least about 90% sequence similarity with the reference sequence. Even more preferably, the aforementioned hydrolase has at least three of said proteolytic activities and at least about 90% sequence identity with the reference sequence. Yet even more preferably, the aforementioned hydrolase has at least three of said proteolytic activities and at least about 95% sequence similarity with the reference sequence.

In embodiments directed to the prevention of adhesions, the inventive method can include pretreatment or simultaneous treatment, or both, of the traumatized membrane with corticosteroids, such as cortisone, alone or in combination with an antihistamine.

As noted above, the multifunctional hydrolase used in the context of the present invention preferably is a krill-derived hydrolase, such as a proteinase. More preferably, the multifunctional hydrolase is part of a multifunctional protein, which may have non-enzymatic functions as well as enzymatic functions. Crustaceans, including antarctic krill, are useful sources for the multifunctional protein of the invention. A protein having "multifunctional activity," is defined herein as including at least one of a chymotrypsin, trypsin, collagenase, elastase or exo peptidase activity, or asialo GM_1 ceramide binding activity. For purification of krill-derived multifunctional protein, see below and, for example, U.S. patent application Serial No.08/600,273 (filed February 8, 1996), deFaire et al., inventors, entitled "Multifunctional Enzyme," which is incorporated herein by reference.

For topical treatments, including treatments to internal surfaces, a preferred suitable dose of multifunctional krill-derived protein per application ranges from about 0.01 U/ml to about 10 U/ml, where typically a layer of from 0.5 to 5 mm of carrier such as cream, ointment, microgel or the like is applied, more preferably about 0.01 U/ml to about 1.0 U/ml, still more preferably about 0.2 U/ml. This dosage range applies to vehicles such as gels, ointments, creams, liquids, sprays, aerosols, and the like. In some embodiments, such as wound debridement, larger dosages may be used initially. Lozenges preferably are designed to deliver about 0.01 U to about 10 U, more preferably about 0.01 U to about 1.0 U, still more preferably about 0.2 U. For all external treatments, the protein composition will generally

be applied from about 1 to about 10 times per day, preferably from about 2 to about 5 times per day. These values, of course, will vary with a number of factors including the type and severity of the disease, and the age, weight and medical condition of the patient, as will be recognized by those of ordinary skill in the medical arts. It is believed that substantially higher doses can be used without substantial adverse effect. Generally, the multifunctional protein will be administered in an effective amount.

Humans are the preferred subjects for treatment. However, the multifunctional protein can be used in many veterinary contexts to treat animals, preferably to treat mammals, as will be recognized by those of ordinary skill in light of the present disclosure.

The composition to be administered is preferably buffered to a physiologically suitable pH, such as pH 6.5 to pH 7.5. Where an enzyme is included in the composition, salts and stabilizing agents can be added in amounts effective to increase activity or stabilize the enzyme.

In another preferred embodiment, the multifunctional hydrolase used in the context of the present invention preferably has the aforementioned proteolytic activity and at least about 60% sequence identity or similarity with a reference sequence. More preferably, the multifunctional hydrolase has at least about 70% identity or similarity with the aforementioned reference sequence; yet more preferably, at least about 80% or 85% identity or similarity with the aforementioned reference sequence; even more preferably, at least about 90% or 95% identity or similarity with the aforementioned reference sequence; and most preferably, at least about 97% identity or similarity with the aforementioned reference sequence. While the percentage similarity noted above is preferred, the percentage identity is more preferred.

Many other administration vehicles are apparent to the artisan of ordinary skill, including, without limitation, slow release formulations, liposomal formulations and polymeric matrices.

The method of treatment of trauma by administering the microgel, with or without the multifunctional hydrolase or other agents, such as the aforementioned antibiotic, is preferably conducted for a suitable time, the suitability of which will be known to the skilled practitioner from inspection of the affected tissue and the kind and severity of the condition being treated. The treatment is preferably administered at least until healing of the affected wound is complete, more preferably for at least an additional five days thereafter. Corneal wounds may be treated for 2 to 35 days. In other cases, the treatment is conducted for at least about 10 days, more preferably for at least about 20 days, yet more preferably for at least about 28 or 35 days. Treatment of cutaneous wounds with a composition containing a microgel and a multifunctional hydrolase can be from 7 to 42 days. Treatments are preferably accomplished via application at least once per day, more preferably twice a day up to about six times a day, using methods of topical application to the eye as are known in the art.

The aforementioned multifunctional hydrolase has a preferred molecular weight of from about 20 kd to about 40 kd; more preferably, the molecular weight is from about 26 kd to about 32 kd.

Preferred multifunctional hydrolases include, but are not limited to *Panaeus vanameii* 1, *Panaeus vanameii* 2, *Panaeus monodon* chymotryptic-1, *Panaeus monodon* tryptic, *Panaeus monodon* chymotryptic-2, *Uca pugilator* enzyme I, *Uca pugilator* enzyme II, Kamchatka crab IA, Kamchatka crab

IIA, Kamchatka crab IIB, Kamchatka crab IIC, Crayfish protease 1, Salmon enzyme 1, Atlantic cod I or Atlantic cod II. Preferably, the aforementioned specific enzymes comprise the following respective peptide sequences: *Panaeus vanameii* 1, I-V-G-G-V-E-A-T-P-H-S-W-P-H-Q-A-A-L-F-I-D-D-M-Y-F (SEQ ID NO:13); *Panaeus vanameii* 2, I-V-G-G-V-E-A-T-P-H-S-X-P-H-Q-A-A-L-F-I (SEQ ID NO:14); *Panaeus monodon* tryptic I-V-G-G-T-A-V-T-P-G-E-F-P-Y-Q-L-S-F-Q-D-S-I-E-G-V (SEQ ID NO:15); *Panaeus monodon* chymotryptic-1; I-V-G-G-V-E-A-V-P-G-V-W-P- Y-Q-A-A-L-F-I-I-D-M-Y-F (SEQ ID NO:16); *Panaeus monodon* chymotryptic-2, I-V-G-G-V-E- A-V-P-H-S-W-P-Y-Q-A-A-L-F-I-I-D-M-Y-F (SEQ ID NO:17); *Uca pugilator* enzyme I, I-V-G-G-V-E-A-V-P-N-S-W-P-H-Q-A-A-L-F-I-D-D-M-Y-F (SEQ ID NO:18); *Uca pugilator* enzyme II, I-V-G-G-Q-D-A-T-P-G-Q-F-P-Y-Q-L-S-F-Q-D (SEQ ID NO:19); Kamchatka crab **IA**, I-V-G-G-Q-E-A-S-P-G-S-W-P-X-Q-V-G-L-F-F (SEQ ID NO:20); Kamchatka crab **IIA**, I-V-G-G-T-E-V-T-P-G-E-I-P-Y-Q-L-S-L-Q-D (SEQ ID NO:21); Kamchatka crab **IIB**, I-V-G-G-T-E-V-T-P-G-E-I-P-Y-Q-L-S-F-Q-D (SEQ ID NO:22); Kamchatka crab **IIC**, I-V-G-G-S-E-A-T-S-G-Q-F-P-Y-Q-X-S-F-Q-D (SEQ ID NO:23); Crayfish protease 1, I-V-G-G-T-D-A-T-L-G-E-F-P-Y-Q-L-S-F-Q-N (SEQ ID NO:24); Salmon enzyme 1, I-V-G-G-Y-E-C-K-A-Y-S-Q-A-Y-Q-V-S-L-N-S-G-Y-H-Y-C (SEQ ID NO:25); Atlantic cod I, I-V-G-G-Y-E-C-T-K-H-S-Q-A-H-Q-V-S-L-N-S-G-Y-H-Y-C (SEQ ID NO:26); or Atlantic cod II, I-V-G-G-Y-E-C-T-R-H-S-Q-A-H-Q-V-S-L-N-S-G-Y-H-Y-C (SEQ ID NO:27).

The most preferred multifunctional hydrolase used in the context of the present invention is PHM-101, which is a purified preparation of a krill multifunctional hydrolase.

The inventive method can include pretreatment or simultaneous treatment, or both, of the affected tissue with a suitable antibiotic. A suitable antibiotic is one that retains its potency when placed in physiological conditions. Some antibiotics are preferred for topical use on tissue, such as, but not limited to ciprofloxacin. The antibiotic can be included in the treatment using the microgel with or without the multifunctional hydrolase.

The present invention provides uses for nucleic acids (such as ribonucleic acids or deoxyribonucleic acids) and polypeptides and analogs thereof, including nucleic acids that bind to a nucleic acid encoding a multifunctional protein, as well as pharmaceutical compositions, gene therapy and antibodies and antisera against the multifunctional protein. Some of the nucleic acids and polypeptides are naturally occurring variants (isoforms) whereas others are non-naturally occurring (i.e., engineered) variants.

1. Nucleic Acids

The nucleic acid sequences disclosed herein encode multifunctional proteins used in the context of the present invention. It is contemplated that the disclosed nucleic acids are useful in the generation of the aforementioned multifunctional proteins. Accordingly, such nucleic acids are preferably deoxyribonucleic acids (DNAs), both single- and double-stranded, and most preferably double-stranded

deoxyribonucleic acids. However, they can also be, without limitation, ribonucleic acids (RNAs), as well as hybrid RNA:DNA double-stranded molecules.

Nucleic acids encoding a multifunctional protein include all multifunctional protein-encoding nucleic acids, whether native or synthetic, RNA, DNA, or cDNA, that encode a multifunctional protein, or the complementary strand thereof, including but not limited to nucleic acid found in a multifunctional protein-expressing organism. For recombinant expression purposes, codon usage preferences for the organism in which such a nucleic acid is to be expressed are advantageously considered in designing a synthetic multifunctional protein-encoding nucleic acid.

The nucleic acid sequences of the invention can encode, for example, one of several isoforms of a krill-derived protein. SEQ ID NOs:2, 4, and 6 represent three isoforms that share about 88-89% identity with each other in overlapping amino acids. See, for example, Figure 1 which compares the DNA sequence of the first isoform, SEQ ID NO:1, with the DNA sequence of the second isoform, SEQ ID NO:3, which share about 88% identical nucleotides. See also, for example, Figure 3, which provides a comparison of the DNA sequence of the third isoform (SEQ ID NO:5) and the first isoform (SEQ ID NO:1), which share about 89% identical nucleotides.

These isoforms all lack the initiation codon methionine. Further, two of these three isoforms contain a hydrophobic sequence which may function as a signal sequence, namely, LLLALVAAASA (SEQ ID NO:28), which is amino acid residues 1-11 in the first isoform, SEQ ID NO:2, and PGRSRIALLLALVAATASA (SEQ ID NO:29), which is amino acid residues 1-19 in the third isoform, SEQ ID NO:6. The features that are important to function as a "signal" peptide which participates in translocating a nascent polypeptide through membrane are well characterized (*see*, for example, von Heijne, *Curr. Opin. Cell Biol.* 2: 604-608, 1990; von Heijne, *J. Mol. Biol.* 184: 99-105, 1985; von Heijne, *J. Mol. Biol.* 173: 243-251, 1984), so that recombinant methods can be used to improve signal peptide function if necessary. The sequences from these von Heijne publications which can be switched with or substituted into the hydrophobic leader sequences of the present invention are hereby incorporated by reference.

These two isoforms additionally contain a pro-protein segment. The pro-protein segment is the segment of the protein, other than the hydrophobic segment, that is present in the precursor protein but absent in the mature protein. Without being limited to a particular theory, it is possible that at least a part of the pro-protein segment may still be attached to the mature protein. Further, it is believed that krill-derived multifunctional proteins may have two chains linked by a disulfide bond. For example, a cysteine in the pro-protein segment may participate in a disulfide bond in the mature protein.

In the first isoform, the pro-protein segment has, for example, the following sequence, which corresponds to amino acid residues 12-63 in the first isoform, SEQ ID NO:2:
AEWRWQFRHPTVTPNPRAKNPFRVTKSSPVQPPAVRGTKAVENCGPVAPRNK (SEQ ID NO:30).
The third isoform has a pro-protein segment with the following sequence, which corresponds to amino acid residues 20-71 in SEQ ID NO:6:

SEWRWQFRHPTVTPNPRANNPFRPSKVAPVQPPAVRGTKAVENCGPVAPKNK (SEQ ID NO:31). The remaining amino acid sequences of these polypeptides (other than the hydrophobic segment and the pro-protein segment) represent the mature protein. See Figure 2, which provides a comparison of the amino acid sequence of the first isoform and the second isoform, which share about 89% identical amino acids. Additionally, see Figure 4 which provides a comparison of the amino acid sequences of all three isoforms.

Further embodiments of the invention include nucleic acid sequences that encode polypeptides that are preferably present in the protein. The following examples are derived from the pro-protein segment of SEQ ID NO:2, and are polypeptides that are preferably present in the mature protein. Without being limited to a particular theory, these polypeptides may form at least part of a first amino acid chain that is linked via a disulfide bond to a second amino acid chain, which can be, for example, the mature protein. For instance, in certain preferred embodiments, the nucleic acid further encodes a polypeptide sequence such as AVENCGPVAPR (SEQ ID NO:32), AVENCGPVAPRNK (SEQ ID NO:33), GTKAVENCGPVAPR (SEQ ID NO:34), GTKAVENCGPVAPRNK (SEQ ID NO:35), SSPVQPPAVRGTKAVENCGPVAPR (SEQ ID NO:36), SSPVQPPAVRGTKAVENCGPVAPRNK (SEQ ID NO:37), or AVENCGPVA (SEQ ID NO:38), or a sequence differing therefrom as indicated in the corresponding sequence fragments of one of the reference sequences. Without being limited to a particular theory, the above-listed polypeptides (SEQ ID NOS:32-38) may be linked to the remainder of the mature krill-derived multifunctional protein via a disulfide bond. For example, the cysteine residue in one of these sequences (SEQ ID NO:32-38) may participate in a disulfide bond with, for example, a cysteine in the mature protein, such as a cysteine corresponding the cysteine at residue 171 of SEQ ID NO:2. At least one of these sequences (SEQ ID NO:32-38) are therefore present in preferred embodiments of the invention. See, for example, Figure 5, which shows the amino acid sequences of several proteins, namely, Factor VII, thrombin, kallikrein, a *Limulus* pro-clotting enzyme from the Japanese horseshoe crab (*Tachypleus tridentatus*), plasmin, hepsin and Factor XII, aligned with the amino acid sequence of SEQ ID NO:2. All of the proteins aligned with the krill-derived multifunctional protein, except for the *Limulus* protein and Hepsin, are involved in the human blood coagulation pathway.

Without being limited to any particular theory, it is believed that krill-derived multifunctional proteins include a somewhat larger N-terminus than that found in SEQ ID Nos:2, 4, 6, 8, 10 or 12. However, as illustrated by the Examples, a functional enzyme can be constructed from the sequences listed herein.

The nucleic acids of the invention can encode engineered multifunctional proteins based on forming chimeric polypeptides from the above isoforms, for example. The hydrophobic sequence or the pro-protein segment of one naturally occurring isoform can optionally be matched with the mature protein sequences of another naturally occurring isoform or isoforms. For example, the mature protein segment of SEQ ID NO:2 is believed to be amino acids 64-300. SEQ ID NO:4, for instance, is a partial sequence of the second isoform, which has a mature protein sequence of about 75% of the length of the

mature protein segment of SEQ ID NO:2. Therefore, certain embodiments of the invention include chimeric polypeptides in which segments from one or more of the described polypeptides are exchanged with the aligned segments of another. For example, the N-terminus of the polypeptide of SEQ ID NO:4 is linked to the remaining 25% of the length of the mature protein sequence found in SEQ ID NO:2, namely amino acids 64-116. In another embodiment of the invention, a hypothetical chimeric sequence includes the first 63 amino acids of the protein of SEQ ID NO:2 together with the amino acid sequence of SEQ ID NO:4. See, for example, Figure 2, which aligns SEQ ID NO:2 with SEQ ID NO:4.

For example, one of the polypeptides can be modified by adding to the N-terminal peptide sequence such as MPGRSRIALLLALVAATASA (SEQ ID NO:39) or $M(X)_nPGRSRIALLLALVLAATASA$ (SEQ ID NO:40) or MPGRSRIALLLALVAAASA (SEQ ID NO:48) or $M(X)_nPGRSRIALLLALVLAAASA$ (SEQ ID NO:49), where n is an integer from 1 to 16, preferably 1 to 12, alternatively 1 to 6, and each X independently represents an amino acid selected to conform to the characteristics of a hydrophobic leader sequence. Any other functional signal sequence, such as any of those referenced in von Heijne, *J. Mol. Biol.* 184: 99-105, 1985, could also be used to make an export version of a protein of the invention. The sequence located after "ASA" allows, in on preferred embodiment, the signal sequence to be cleaved during cellular processing. Note that SEQ ID NOs: 39 and 40, which are derived from leader sequence found in SEQ ID NOs: 2, 6, 8, 10 and 12, have the minimal characteristics described in von Heijne, *J. Mol. Biol.* 184: 99-105, 1985: a c-region in residues -1 to -5; an h-region of at least seven residues in the -6 to -14 sequence, with no Ser, Gly, Thr or Pro residue among the hydrophobic residues in the h-region (though as many as one can often be acceptable); and a positively charged n-region of at least one residue. The n-region tends to have 1.7 positively charged residues on average, where, consistent with this rule, that of the above described sequences has 2 such residues. These regions are illustrated below by sequence derived from SEQ ID NOS: 2 and 6:

```
MPGRSR IALLLALV AATASA SerGluTrpArgTrp          (SEQ ID NO:50)
MPGRSR IALLLALV AA ASA AlaGluTrpArgTrp          (SEQ ID NO:51)
       └────┘ └──────┘ └────┘
       n-region h-region  c-region
```

Table 2 of von Heijne, *J. Mol. Biol.* 184: 99-105, 1985 illustrates how well the above sequence comports with other signal sequences that have been identified. Other preferred cleavage-associated properties apply to these sequences. See, von Heijne, *J. Mol. Biol.* 173: 243-251, 1984. For instance, cleaved sequences have a strong preponderance of Ala at positions -1 and -3, as above. Large polar residues and charged residues are enriched in the +1 to +5 sequence, as above. Alanine is markedly reduced at positions -2, +2 and +5, as above.

Without being bound to a particular theory, it is believed that there are at least about 4-5 isoforms, each having a different amino acid at the position corresponding to amino acid residue 68 of SEQ ID NO:2, including glutamine, methionine, lysine and asparagine. Such isoforms and other homologous polypeptides can be isolated using the techniques described below.

To construct engineered variants of multifunctional protein-encoding nucleic acids, the native sequences of any of the isoforms can be used as a starting point and modified to suit particular needs. For example, in certain embodiments, the nucleic acid sequence need not include the sequences encoding the 5' portion of the amino acid sequence that is absent in the mature protein, including amino acids 1-63 of SEQ ID NO:2. Thus, in certain embodiments of the invention, the encoded polypeptide is homologous to or has the sequence of the mature protein only, and not the segments corresponding to the N-terminal portions that are removed during cellular processing, namely, the hydrophobic sequence and the pro-protein segment.

Nonetheless, in preferred embodiments of the nucleic acids of the invention, the sequences encoding the N-terminal portion of the amino acid sequence that is absent in the mature protein, including amino acids 1-63 of SEQ ID NO:2, are included in the nucleic acid sequences.

The amino acid sequence forming a synthetic multifunctional protein preferably includes an enzymatically active segment of a krill-derived multifunctional protein, such as amino acids 64-300 of SEQ ID NO:2, particularly including the histidine at residue 104, the aspartic acid at residue 151 and the serine at residue 246, which are implicated in the catalytic mechanism of serine proteases. Thus, the protein need not include the hydrophobic sequence or pro-protein segment that are present in a krill-derived protein before cellular processing occurs, although the hydrophobic sequence and the pro-protein segment are preferably present.

Preferably, the nucleic acids will encode polypeptides having at least about 60% identity or similarity, more preferably, at least about 80% identity or similarity, even more preferably, at least about 85% identity or similarity, yet more preferably at least about 90% identity or similarity, and most preferably at least about 95% identity or similarity to a reference protein or a krill-derived multifunctional protein, such as the polypeptides of SEQ ID NOs:2, 4, 6, 8, 10 or 12 or amino acid sequences 64-300 of SEQ ID NO:2, or other naturally occurring isoforms or reference sequences. Even more preferably, the nucleic acids will encode polypeptides sharing at least about 60% identity, more preferably, at least about 70% identity, yet more preferably, at least about 80% identity, still more preferably at least about 85% identity, yet still more preferably at least about 90% identity and most preferably at least about 95% identity with a krill-derived multifunctional protein or a reference sequence.

Additionally, the invention includes an isolated nucleic acid comprising a nucleic acid that binds to a nucleic acid encoding a polypeptide having at least about 70% identity or similarity to a reference protein or a krill-derived multifunctional protein. Even more preferably, the nucleic acid binds to a nucleic acid encoding a polypeptide having at least about 80% identity or similarity, and more preferably, at least about 90% identity or similarity to a krill-derived multifunctional protein. Yet more preferably, the nucleic acid binds to a nucleic acid encoding a polypeptide sharing at least about 70% amino acid identity, and more preferably, at least about 80% amino acid identity, and yet more preferably, at least about 90% amino acid identity with a krill-derived multifunctional protein, or a polypeptide of one of the reference sequences. A nucleic acid that binds to a nucleic acid that encodes a polypeptide homologous to

a krill-derived multifunction protein can be used as a probe, for example, to identify additional multifunctional proteins or to determine multifunctional protein expression.

The mature protein of the polypeptide of SEQ ID NO:2 is about 61% identical to the chymotrypsin-like serine proteinase in the shrimp *Penaeus vannamei* according to the sequence provided by Genbank (Mountain View, CA), database acquisition no. X66415, and about 60% identical to the collagenolytic serine proteinase in the fiddler crab *Uca pugilator*, according to the sequence provided by Genbank, database acquisition no. U49931. The amino acid sequence of the pro-protein of SEQ ID NO:2 is about 53% identical to the precursor of the chymotrypsin-like serine proteinase in the shrimp *Penaeus vannamei*, and about 51% identical to the precursor of the collagenolytic serine proteinase in the fiddler crab *Uca pugilator*. Preferably, the nucleic acids encoding polypeptides having multifunctional activity are less than about 70% identical to the above-identified proteinases of *Penaeus vannamei* or *Uca pugilator*.

In addition to nucleic acids encoding a multifunctional protein, the present invention includes nucleic acids encoding polypeptides that are homologous to a reference protein or a krill-derived multifunctional protein or that share a percentage identity with a reference protein or a krill-derived multifunctional protein. Further, the present invention includes nucleic acids that encode a portion of a multifunctional protein or a variant thereof, such as the enzymatically active portion of the protein or the portion of the protein that provides asialo GM_1 ceramide binding activity.

The invention also is directed to a nucleic acid encoding a krill-derived multifunctional protein that has at least one of the following activities: chymotrypsin, trypsin, collagenase, elastase and exopeptidase activity or asialo GM_1 ceramide binding activity. Preferably, the encoded polypeptide will be effective to remove or inactivate a cell-surface adhesion molecule, and most preferably, the encoded polypeptide will be pharmaceutically effective.

For identifying the active segment or segments of multifunctional protein, one approach is to take a multifunctional protein cDNA and create deletional mutants lacking segments at either the 5' or the 3' end by, for instance, partial digestion with S1 nuclease, Bal 31 or Mung Bean nuclease (the latter approach described in literature available from Stratagene, San Diego, CA, in connection with a commercial deletion cloning kit). Alternatively, the deletion mutants are constructed by subcloning restriction fragments of a multifunctional protein cDNA. The deletional constructs are cloned into expression vectors and tested for their multifunctional activity.

These structural genes can be altered by mutagenesis methods such as that described by Adelman et al., *DNA*, 2: 183, 1983 or through the use of synthetic nucleic acid strands. The products of mutant genes can be readily tested for multifunctional activity.

The nucleic acid sequences can be further mutated, for example, to incorporate useful restriction sites. See Maniatis et al. *Molecular Cloning, a Laboratory Manual* (Cold Spring Harbor Press, 1989). Such restriction sites can be used to create "cassettes", or regions of nucleic acid sequence that are facilely

substituted using restriction enzymes and ligation reactions. The cassettes can be used to substitute synthetic sequences encoding mutated multifunctional protein amino acid sequences.

The multifunctional protein-encoding sequence can be, for instance, substantially or fully synthetic. See, for example, Goeddel et al., *Proc. Natl. Acad. Sci. USA*, 76, 106-110, 1979. For recombinant expression purposes, codon usage preferences for the organism in which such a nucleic acid is to be expressed are advantageously considered in designing a synthetic multifunctional protein-encoding nucleic acid. Since the nucleic acid code is degenerate, numerous nucleic acid sequences can be used to create the same amino acid sequence.

Further, with an altered amino acid sequence, numerous methods are known to delete a sequence from or mutate nucleic acid sequences that encode a polypeptide and to confirm the function of the polypeptides encoded by these deleted or mutated sequences. Accordingly, the invention also relates to a mutated or deleted version of a multifunctional protein nucleic acid that encodes a polypeptide that retains multifunctional protein activity.

Conservative mutations of the naturally occurring isoforms are preferred for engineered variants. Such conservative mutations include mutations that switch one amino acid for another within one of the following groups:

1. Small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr, Pro and Gly;
2. Polar, negatively charged residues and their amides: Asp, Asn, Glu and Gln;
3. Polar, positively charged residues: His, Arg and Lys;
4. Large aliphatic, nonpolar residues: Met, Leu, Ile, Val and Cys; and
5. Aromatic residues: Phe, Tyr and Trp.

A preferred listing of conservative substitutions is the following:

Original Residue	Substitution
Ala	Gly, Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Ala, Pro
His	Asn, Gln
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Tyr, Ile
Phe	Met, Leu, Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe
Val	Ile, Leu

The types of substitutions selected may be based on the analysis of the frequencies of amino acid substitutions between homologous proteins of different species developed by Schulz et al., *Principles of Protein Structure*, Springer-Verlag, 1978, pp. 14-16, on the analyses of structure-forming potentials developed by Chou and Fasman, *Biochemistry* 13, 211, 1974 or other such methods reviewed by Schulz et al. *Principles in Protein Structure*, Springer-Verlag, 1978, pp. 108-130, and on the analysis of hydrophobicity patterns in proteins developed by Kyte and Doolittle, *J. Mol. Biol.* 157: 105-132, 1982.

2. Polypeptides

Polypeptides used in the context of the present invention include all polypeptides having multifunctional activity, whether native or synthetic, including but not limited to polypeptides purified from a multifunctional protein-expressing organism. A preferred embodiment of the invention provides a polypeptide comprising a substantially pure isoform of a reference protein or a krill-derived multifunctional protein or engineered variant thereof, and more preferably, a polypeptide comprising SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10 or SEQ ID NO:12. Further, polypeptides of the invention preferably comprise at least one of the amino acid sequences of SEQ ID NOs:32-38.

In addition to the reference protein or a multifunctional protein, and their isoforms and portions thereof, the present invention includes use of polypeptides that are homologous to a reference protein or a krill-derived multifunctional protein or that share a percentage identity with a reference protein or a krill-derived multifunctional protein. Further, the present invention includes portions of the a reference protein or a multifunctional protein or a variant thereof, such as the enzymatically active portion of the protein or the portion of the protein that provides asialo GM_1 ceramide binding activity.

Additionally, the present invention includes engineered variants of multifunctional proteins that retain multifunctional activity. In certain embodiments, these engineered variants lack, for example, no more than about 63 amino acid residues at the N-terminal end of SEQ ID NO:4.

Preferably, the variants will have at least about 60% identity or similarity, more preferably, at least about 70% identity or similarity, even more preferably, at least about 80% identity or similarity, still more preferably at least about 85% identity or similarity, yet still more preferably at least about 90% identity and most preferably at least about 95% identity or similarity to a krill-derived multifunctional protein, such as the polypeptides of the reference sequences, or other isoforms, or amino acid sequences 64-300 of SEQ ID NO:2. Even more preferably, the analogs will share at least about 70% identity, more preferably, at least about 80% identity, yet more preferably, at least about 85% identity, still more preferably at least about 90% identity, and most preferably at least about 95% identity with a polypeptide of a reference sequence.

Preferably, the polypeptide has the sequence of a contiguous stretch of at least about 237 amino acids of mature proteins of SEQ ID NO:2, or of another reference sequence.

Amino acid analogs of the above-described polypeptides are also included in the present invention.

Preferably, the enzyme is simply isolated from a suitable biological source, such as krill. However, other techniques, such as recombinant technology, can be used.

Additionally, the present invention provides a pharmaceutical composition comprising a hydrolytic enzyme such as described in combination with a polymer microgel, the enzyme and the microgel each in a therapeutically effective amount. In the combination, the effective amount of either the enzyme or the microgel can be an amount effective independent of the presence of the other agent (enzyme or microgel) or it can be an amount by which one agent enhances the effectiveness of the other, with the overall the combination being present in an effective amount.

3. Preferred Purification Methods

The present invention also provides for a novel method of isolating the multifunctional protein used in the aforementioned methods for treating trauma to epithelia from the sea water crustaceans from which it is derived. Preferably, the source of the multifunctional hydrolase is krill, although as set forth elsewhere herein, other aquatic (preferably crustacean) sources have been identified. The method of isolating the hydrolase can comprise extracting protein from the animals, such as extracting in fresh water, that is water having a substantially lower osmolarity than has sea water, such as but not limited to potable water, preferably for between about ten minutes and about three hours, more preferably for about 30 minutes and about two hours, yet more preferably for about 45 minutes and about 90 minutes. The crude extract can then preferably be separated from the insoluble material via standard means, such as via one or more of sedimentation, centrifugation, filtration, and the like. The clarified crude extract is then preferably subjected to a method to remove any remaining particulate matter, such as virus particles and cell debris that may have been co-extracted from the source animals, such as via a microfiltration method. The further clarified crude extract is then preferably subjected to various methods of standard protein purification procedures, such as differential filtrations, differential precipitations, ion exchange chromatography, affinity chromatography, molecular sieving, and the like. A preferred method for isolating the multifunctional hydrolase used in the context of the present invention includes the use of an affinity column that includes a ligand that is suitable for binding to the multifunctional protein. In particular, the ligand is preferably a boronate derivative, more preferably a phenylboronate species, yet more preferably an aminophenylboronate species, which can be substituted or not. For example, it is contemplated that the ligand used in the context of the present method of isolating the multifunctional hydrolase can have other moieties attached thereto, including but not limited to in each instance alkyl (C_1-C_8), aryl (C_5-C_7), chloro, fluoro, alkoxy (C_1-C_8), and other such moieties, so long as the moieties do not substantially interfere with the specific binding of the multifunctional hydrolase derived from krill, for example, as set forth herein. By substantially interfering with the specific binding, it is intended that the aforementioned added species do not substantially interfere with the said specific binding to the extent that such binding is not reduced thereby by more than about 20%, more preferably, by not more than about 10%.

Additionally, the present invention provides a pharmaceutical composition for treating an animal comprising an effective amount of a polypeptide comprising a substantially pure isoform of a krill-derived multifunctional protein or engineered variant thereof and a pharmaceutically acceptable carrier, especially a carrier that is a microgel. More preferably, the polypeptide comprises a reference sequences, and the polypeptide preferably comprises at least one of the amino acid sequences of SEQ ID NO:32-38.

4. Methods of Synthesizing Polypeptides

In one embodiment, the polypeptides of the invention are made as follows, using a gene fusion. For example, fusion to maltose-binding protein ("MBP") can be used to facilitate the expression and purification of a multifunctional protein in a prokaryote such as *E.coli*. The hybrid protein can be purified, for example, using affinity chromatography using the binding protein's substrate. See, for example, *Gene* 67: 21-30 (1988). When using a fusion polypeptide that includes maltose binding protein, a cross-linked amylose affinity chromatography column can be used to purify the protein.

The cDNA specific for a given multifunctional protein or analog thereof can also be linked using standard means to a cDNA for glutathione s-transferase ("GST"), found on a commercial vector, for example. The fusion polypeptide expressed by such a vector construct includes the multifunctional protein or analog and GST, and can be treated for purification.

Should the MBP or GST portion of the fusion polypeptide interfere with function, it is removed by partial proteolytic digestion approaches that preferentially attack unstructured regions, such as the linkers between MBP or GST and the multifunctional protein. The linkers can be designed to lack structure, for instance using the rules for secondary structure-forming potential developed by Chou and Fasman, *Biochemistry* 13, 211, 1974. The linker is also designed to incorporate protease target amino acids, such as for trypsin, arginine and lysine residues. For example, the linker can incorporate the Asp-Asp-Asp-Asp-Lys (SEQ ID NO:41) target sequence of bovine enter okinase (recombinant subunit available for example from Invitrogen, Carlsbad, CA). To create the linkers, standard synthetic approaches for making oligonucleotides are employed together with standard subcloning methodologies. Other fusion partners other than GST or MBP can also be used. For example, the fusion polypeptide can be as found in vectors available from Invitrogen, such as TRX-Fus vectors that incorporate thioredoxin as the fusion polypeptide.

Additionally, the multifunctional proteins can be directly synthesized from nucleic acid (by the cellular machinery) without use of fusion partners. For instance, nucleic acids having the sequence of SEQ ID NOs:1, 3, 5, 7, 9 or 11 are subcloned into an appropriate expression vector having an appropriate promoter and expressed in an appropriate organism. Antibodies against the krill multifunctional protein can be employed to facilitate purification.

Additional purifications techniques are applied as needed, including without limitation, preparative electrophoresis, FPLC (Pharmacia, Uppsala, Sweden), HPLC (e.g., using gel filtration, reverse-phase or mildly hydrophobic columns), gel filtration, differential precipitation (for instance,

"salting out" precipitations), ion-exchange chromatography and affinity chromatography (including affinity chromatography using the RE1 duplex nucleotide sequence as the affinity ligand).

A polypeptide or nucleic acid is "isolated" in accordance with the invention in that the molecular cloning of the nucleic acid of interest, for example, involves taking a multifunctional protein nucleic acid from a cell, and isolating it from other nucleic acids. This isolated nucleic acid may then be inserted into a host cell, which may be yeast or bacteria, for example. A polypeptide or nucleic acid is "substantially pure" in accordance with the invention if it is predominantly free of other polypeptides or nucleic acids, respectively. A macromolecule, such as a nucleic acid or a polypeptide, is predominantly free of other polypeptides or nucleic acids if it constitutes at least about 50% by weight of the given macromolecule in a composition. Preferably, the polypeptide or nucleic acid of the present invention constitutes at least about 60% by weight of the total polypeptides or nucleic acids, respectively, that are present in a given composition thereof, more preferably about 80%, still more preferably about 90%, yet more preferably about 95%, and most preferably about 100%. Such compositions are referred to herein as being polypeptides or nucleic acids that are 60% pure, 80% pure, 90% pure, 95% pure, or 100% pure, any of which are substantially pure.

5. Preferred Characteristics of the Multifunctional Protein

Krill, including without limitation krill of the genuses *Euphausia* (such as *superba, crystallorphias, frigida, triacantha, vellantini, lougirostris, lucens, similis, spinifera, recurva* and the like), *Meganyctiphanes* (such as *norvegica* and the like) and *Tysanoessa* (such as *macurura, vicina, gregaria* and the like), are a preferred source of krill-derived multifunctional proteins.

Preferably, the protein has a molecular weight between about 20 kd and about 40 kd, and more preferably from about 26 kd to about 32 kd, and most preferably about 29 kd, as determined by sodium dodecyl sulfate ("SDS") polyacrylamide gel electrophoresis ("PAGE"). Further, the protein preferably has substantial homology to a krill-derived multifunctional protein. Preferred proteins are hydrolases, and preferably, proteases. Preferably, the protein is selectively reactive with cell-surface receptors such as polypeptides or glycolipids.

Protease activity can be determined by incubating a protein preparation with casein (concentration 1% w/v) for example at 30°C, and measuring the release of amino acids or peptides (which can be measured by the increase in colorometrically determinable amino groups). Casein Units are defined in *Biochem. J.*, 173: 291-298, 1978 (using azocasein as the substrate).

Alternatively, tryptic protease activity can be measured against tyrosine-arginine-methyl-ester ("TAME"). Or, tryptic activity can be measured using Benzoyl-Val-Gly-Arg-p-NO_2-anilide as the substrate, for example, using the method of *Biochemical J.*, 185: 423-433, 1980. Chymotryptic activity can be measured using Succinyl-Ala-Ala-Pro-Phe-p-NO_2-anilide as the substrate, and the method of *J. Biol. Chem.*, 269: 19565-19572, 1994. Elastase activity can be measured using Boc-Ala-Ala-Pro-Ala-p-NO_2-anilide as the substrate, and the method of *J. Biol. Chem.*, 269: 19565-19572, 1994.

When HL60 cells are pretreated with krill-derived multifunctional hydrolase, their binding to TNF stimulated endothelial cells is inhibited by more than about 60%. Preferably, treatment of HL60 or endothelial cells with the multifunctional protein of the invention will inhibit HL60 cell binding to TNF stimulated endothelial cells by at least about 20%, more preferably at least about 40%, still more preferably at least about 60%, yet more preferably at least about 80%. Alternately, the multifunctional protein will preferably have at least about 30% of the adhesion-inhibiting activity of the krill-derived multifunctional hydrolase. More preferably, the multifunctional protein shall have at least about 60% of the adhesion inhibiting activity of the krill-derived multifunctional hydrolase, still more preferably at least about 80%, yet more preferably at least about 100%.

The multifunctional protein of the invention effectively removes or inactivates certain cell-surface adhesion molecules, such as ICAM-1 (i.e., CD 54), ICAM-2, VCAM-1, CD4, CD8, CD28, CD31, CD44 and the asialo GM_1 ceramide, without affecting cell viability. This adhesion site removal or inactivation phenomenon is believed to provide at least a partial explanation for the protein's effectiveness against many, though probably not all, of the indications against which the multifunctional protein is effective as a treatment or preventative agent. Other cell surface receptors have been found to be substantially resistant to removal or inactivation by the multifunctional protein, such as the T-cell receptor, the Class I major histocompatibility complex or the integrins CD11 and CD18.

The hydrolase used with a polyanionic polymer to treat a wound is preferably a protease that digests one or more of certain cell-surface adhesion molecules, such as ICAM-1 (i.e., CD 54), ICAM-2, VCAM-1, CD4, CD8, CD28, CD31, CD44 and the asialo GM_1 ceramide, without affecting cell viability. Preferably, the protease digests at 2, 3, 4, 5, 6, 7, 8 or all of these cell-surface adhesion molecules. In particular, the protease is preferably a protease, especially a multifunctional protease such as described above.

Example 1. Cloning of PHIM Polypeptide

The PHIM polypeptide was purified and the polypeptide was partially sequenced, as described in U.S. patent application Serial No. 08/600,273 (filed February 8, 1996), deFaire et al., inventors, entitled "Multifunctional Enzyme." Degenerate oligonucleotide primers were constructed based on the partial amino acid sequence. The primers had the following sequences: CACGCCTACCCITGGCA (SEQ ID NO:42) and GTGTTGGACTCGATCCAGATC (SEQ ID NO:43). The primers were used to screen a krill cDNA library that was constructed in lambda zap, using the lambda zap cDNA synthesis kit (Stratagene, San Diego, CA). Three positive clones were identified through screening with a PCR fragment as a probe. The PCR fragment used as a probe was sequences 217 to 881 of SEQ ID NO:1, with the following changes: at 219, T to C; at 222, T to C; at 228, C to G; at 270, T to A; at 330, G to A; at 417, C to A; at 534, T to C; at 741, C to T; and at 825, C to G. The three positive clones were sequenced.

the first clone resulting in SEQ ID NO:1, the second clone resulting in SEQ ID NO:2 and the third clone resulting in SEQ ID NO:7. These isoforms all lack the initiation codon methionine.

Example 2. Expression of Recombinant Multifunctional Protein Enzyme

A recombinant multifunctional protein was expressed in an *E.coli* as follows, using the BamHI and Xho I sites of a pET23c vector provided by Novagen (Abingdon, Oxford, U.K.). The pET23c vector includes a gene10 tag for facilitating purification of the expressed recombinant protein. Further, the pET vector places the recombinant multifunctional protein under the control of bacteriophage T7 transcription and translation signals. Once established in a non-expression host, *E.coli* MC1061, the plasmid was then transferred to an expression host, *E.coli* BL21.(DE3) pLYS S having a chromosomal copy of the T7 polymerase gene under lacUV5 control. Expression was induced by the addition of 1 mM IPTG at an optical density of 0.5 at wavelength 600. The cells were harvested after 2 hours at an optical density of 1.0. The recombinant protein was insoluble in the lysate and after harvesting, it was washed and dissolved in 6 M urea. Refolding of the recombinant protein was carried out by 200-fold dilution using a buffer containing 100 mM tris HCl pH 9.5, 100 mM $CaCl_2$, 0.3 mM oxidized glutathione and 3 mM reduced glutathione, followed by stirring overnight at 4°C.

Example 3.

A fragment from the p62 clone that encodes amino acids 56 to 300 and the stop codon (see SEQ ID NOs:1 an 4) was excised and inserted it into a pET-23c expression vector. Note that this clone excludes the cysteine required to form a disulfide link to a light chain. The encoded protein was expressed in *E.coli* strain BL21(DE3)pLysS, which yielded an insoluble material. The insoluble material was dissolved in 6 M urea, and re-folded by a 200-fold dilution into an aqueous solution buffered at pH 9.5, containing 0.1 mM CaCl and oxidized/reduced glutathione. The resulting solution was concentrated to recover the recombinant protein.

The recombinant protein was shown to cleave the model substrate succinyl-ala-ala-pro-phe-*p*-nitroanilide, thereby demonstrating its proteolytic activity. The proteolytic activity was inhibited by the protease inhibitor Eglin.

Example 4.

The multifunctional hydrolase derived from krill that is used in the context of the present invention is obtained from the antarctic krill species, Euphausia superba. A preferred procedure for extraction, purification and formulation into a bulk active formulation is achieved by the four step process outlined below. Activity of PHM-101 was monitored in accordance with the procedure set forth at Example 7. The multifunctional hydrolase product resulting from the following procedure has been labeled PHM-101, and displays an N-terminal sequence of I-V-G-G-N/M-E-V-T-P-H-A-Y-P-W-Q-V-G-L-F-I-D-D-M-Y-F (SEQ ID NO:52)

Production of Freeze-dried PHM-101
(Nominal Batch: 100kg Krill)

Step	Material	Activity (U)/Yield (%)	
	Krill		
1 Extraction			
	Microfiltered Extract	200,000	100
2. Anion Exchange Chromatography			
	DEAE Eluate	150,000	75
3. Affinity Chromatography			
	PHM-101 Solution	125,000	83
4. Viral Filter Freeze-dry			
	Freeze-dried PHM-101	100,000	80

Krill Fishing and Supply. The Krill used for the present invention were supplied by the established Japanese fishing company, Nissui. In order to prevent spoiling (including autolysis by the digestive enzymes), on board ship the krill were washed with seawater, packed into plastic bags containing approximately 12.5 kg, placed into labeled boxes indicating the date caught (two bags per box), flash frozen at a temperature of -40°C and so stored until thawed for extraction. In accordance with a preferred aspect, only krill caught in December and January were used as this has been shown to be the time when the digestive enzyme activity is highest and lipid content is lowest, thus making extraction maximally efficient.

PHM-101 Extraction, Purification and Formulation. The production process used standard protein separation and purification techniques and equipment.

Four 25kg boxes of frozen krill were transferred from the bulk freezer store to a chest freezer. After checking that the krill were in good condition, they were removed from their outer boxes, placed in trays and allowed to defrost slowly overnight in the cold room at 4°C. The next morning, the now partly defrosted krill were removed from their plastic wrapping, separated into small lumps and added to 400 litres of potable water in the extraction tank. Here the material was gently stirred for at least an hour until all the ice and lumps disappeared. At various stages in the process samples were taken for analysis

and stored indefinitely or at least until the satisfactory completion of the run. The analyses conducted during the process are outlined below.

The crude extract was then separated from the solid waste by passing it through a coarse, rotating drum sieve. This fine extract was then pumped into a tall insulated sedimentation tank, where it was left overnight to allow the heavier components to sink to the bottom.

The next morning the supernatant was run off into an insulated holding tank, from where it was pumped to the microfiltration rig and the sediment was discarded.

The microfiltration rig used is a standard piece of equipment from Millipore and is specifically designed for pharmaceutical processing. Essentially this rig pumps the supernatant through a 2 micron filter array to remove particulate debris, thus giving a clear solution for application to the first chromatography column. The microfiltered extract was pumped through into a clean room area, where it was stored overnight in an insulated holding tank.

The first purification step in the process was a standard anionic exchange chromatography step, using an axial flow column containing ten litres of standard DEAE-sepharose, purchased from Pharmacia (Uppsala, Sweden). The column was equilibrated using a flow rate of about 120 liters per hour and not less than 5 column volumes of 20 mM Tris-HCl buffer, pH 7.5 (Buffer A). After various standard preparation steps required to place the microfiltered extract into an appropriate volume, concentration, and salt content, the microfiltered extract was pumped onto the equilibrated DEAE-sepharose column using Buffer A, which is designed to selectively bind molecules with overall negative charge. Other unwanted molecules passed through and were discarded after flowing 120 liters per hour with 10 +/- 3 column volumes of 10 mM Tris-HCl, 0.30 M NaCl buffer, pH 7.5 (Buffer B). By then washing the column at a rate of 60 liters per hour with approximately 4 column volumes of 10 mM Tris-HCl, 0.50 NaCl buffer, pH 7.5 (Buffer C), the PHM-101 is selectively displaced and collected from the column in three fractions. This is referred to as the DEAE-Eluate. Other unwanted molecules were left behind. These unwanted molecules were also washed from the column and discarded by flowing 5 column volumes of 10 mM Tris-HCl, 1.00 M NaCl buffer, pH 7.5 (Buffer D), followed by 2 column volumes of 1 M NaOH, at a flow rate of about 120 liters per hour, allowing the column to stand without any flow for about 1 to about 24 hours, and then washing with 10 column volumes of distilled water, finally equilibrating the column with Buffer A as above, making the column ready for re-use. Typically such a column is re-usable ten times before the sepharose is replaced.

The second step in the purification process was an affinity chromatography step, which exploits the substrate-binding preferences of various enzymes for particular chemical groups. The column used consists of a standard axial chromatography column, packed with 500 ml of sepharose to which has been attached a ligand having the structure aminophenylboronate. Alternatively, for example, an agarose chromatography material could be used, such as Agarose 6XL, which is available commercially. Using a flow rate of approximately 250 ml per minute, the column was equilibrated with 10 +/- 5 column volumes of Buffer C. Thereafter, the DEAE-eluate was pumped onto the affinity column which specifically binds

serine-protease enzymes like PHM-101, using a flow rate of approximately 200 ml per minute, and continuing to wash the thus loaded column with 10 +/- 5 column volumes of Buffer C. Contaminants that do not bind to the column and were discarded to waste, including those dislodged by further washing of the column with (1) 10 +/- 5 column volumes of 50 mM Tris-HCl, 5 mM $CaCl_2$ buffer, pH 7.5 (Buffer E), also at a rate of about 200 ml per minute, and (2) 1 +/- 0.8 column volume of 10 mM Glycine-citrate, 1.0 mM $CaCl_2$ buffer, pH 7.4 (Buffer F) at a rate of about 100 ml per minute. By then washing the column with about 4 column volumes of 10 mM Glycine-citrate, 1.0 mM $CaCl_2$, 200 mM Sorbitol buffer, pH 7.4 (Buffer G) at a rate of about 100 ml per minute, PHM-101-containing solution is displaced and collected. Other unwanted molecules are left behind. These unwanted molecules are subsequently washed from the column using the following series of washes: Wash 1, 5 column volumes of 30% v/v 2-propanol, 0.2 M NaOH followed by 5 column volumes of purified water, at 250 ml per minute; Wash 2, 5 column volumes of 0.1 M HCl followed by 5 column volumes of purified water at 250 ml per minute; Wash 3, 5 column volumes of 50 mM NaOH followed by 5 volumes of purified water, at 250 ml per hour; and Wash 4, 5 column volumes of 25% ethanol/75% 0.1 M NaCl, at 250 ml per hour. Typically such a column is re-usable at least ten times before the sepharose is replaced.

To minimize the bioburden of the PHM-101 solution, it was filtered twice in a sterile cabinet using aseptic handling technique. The first is a step designed to remove viruses and uses a commercially available virus filter, namely Planova 15 virus filter designed for the purpose. The second, uses a commercially available 2 micron filter, using a vacuum pump to pass the filtered PHM-101 solution therethrough. Before and after each filtration the integrity of each filter unit was tested and the material was only released if both tests were passed. The filtered solution was aseptically filled into a sterile container and labeled before being removed from the sterile cabinet and stored in a chest freezer reserved for the storage of filtered PHM-101 solution.

Frozen filtered PHM-101 solution was then thawed and aliquoted into vials such that each vial contained 50 Units of PHM-101 activity. These Units are as defined hereinabove. The aliquoted PHM-101 formulation, to which various excipients can be added to protect the enzyme as described elsewhere in the specification, was then frozen and freeze-dried in accordance with standard procedures.

Analyses conducted during the production of freeze-dried PHM-101 were quick in-process analyses, used to control and monitor the effectiveness of the process during each step, as follows:

The activity (of PHM-101) was assayed throughout the process to check the quantity and functionality of the material being produced. Activity was measured by monitoring the rate of reaction with the *p*-nitroanilide ester of succinyl-Ala-Ala-Pro-Phe, a known substrate of chymotrypsin-like enzymes, using a standard assay.

The protein content was measured at various steps in the process by a standard method known as the Bradford method. As enzymes are proteins, this is another means of monitoring the quantity and quality of the material being produced by the process. Dividing the activity number by this yields a useful measure of functionality and purity called the specific activity.

Protein contaminants or peptide breakdown products of the PHM-101 preparation were tested using standard SDS-poly acrylamide gel electrophoresis.

Mono-Q FPLC [Pharmacia] is a commercially available analytical chromatography column which separates the components of a mixture by virtue of their relative charge. An aliquot is analyzed via the column, and eluted protein was detected via ultraviolet adsorption characteristics of the protein. Pure molecules show up as a single, sharp symmetrical peak. Other peaks indicate the presence of contaminants (either impurities or breakdown products).

Example 5.

An embodiment of the present invention directed at a method of treatment of corneal wounds was tested using albino, New Zealand rabbits (2-3 kg), experimentally causing alkaline burn wounds of the corneas of anesthetized rabbits, followed by control and inventive treatments, as set forth herein below:

Forty albino, New Zealand rabbits (2-3 kgs) were anesthetized with intravenous phenobarbital using standard procedures. Filter paper discs, 7 mm, were wetted, in the first instance, with 4N sodium hydroxide solution, and were applied to the central part of the cornea for one minute, with precautions taken to avoid involvement of the peripheral part of the cornea, conjunctiva or lids. The burned corneas were then being irrigated with 0.9% sodium chloride. Epithelium was debrided with a cotton tipped applicator, and ciprofloxacin drops (Ocuflex) instilled.

The animals were randomly divided into two groups and each animal was treated topically for 28 days either with protease PHM-101 (prepared in accordance with Example 4) or a microgel without protease. A masked observer performed daily slit lamp biomicroscopy to document the clinical parameters.

Protease PHM-101 was made up by reconstituting 1 vial with 2 ml sterile water and adding the solution to 10 ml of microgel prepared with Carbopol® 974P according to the procedure in Example 6 to follow. The mixture was shaken to homogenize. The dose was 50 mcL of the reconstituted gel four times daily, applied 10 minutes after the antibiotic. As mentioned above, one group of animals (the gel-only group) was treated with the microgel that did not contain enzyme. Treatment was started on the day following the wounding and continued for 28 days, with a period of observation of 1 week off-treatment in the event of incomplete healing. Post-operative treatment for all 40 rabbits included the use of a topical antibiotic (ciprofloxacin) four times daily until the cornea was re-epithelialized, in order to prevent secondary bacterial infection of the cornea.

The rabbits were observed daily by slit lamp biomicroscopy to document certain clinical parameters, including size of residual epithelial defect, degree of stromal ulceration, and, at the end of healing, degree of stromal opacification. The size of the epithelial defect remaining was assessed by means of a grid in one ocular of the biomicroscope. The degree of ulceration and of stromal opacification (scarring) neovascularisation was assessed on a subjective scale of 0 to 4+ (mild to severe). The

observations were masked such that the observer was not aware of whether a particular rabbit was in the protease or the gel-only group.

On day 28, all the animals with healed epithelial defects were killed and their corneas harvested for histopathological studies. The remaining animals were observed for one week without treatment and were then killed on day 35 to harvest corneas for histopathology.

By day 28, 7/20 of the enzyme treated eyes had corneal ulcers, whilst 6/18 of the placebo-treated eyes had ulcers. By day 35 (one day after termination of treatment) 2/20 of the remaining enzyme-treated corneas were ulcerated, and 6/18 of the placebo-treated corneas were ulcerated. The study did not contain a no-treatment or saline-only treatment group. However, the wealth of published literature on this animal model indicates that in the same model using a 1N alkali burn (a much less severe burn than the 4N alkali burn used in this Example) approximately 75% of the eyes would develop ulcers.

This example provides results that demonstrate the benefit of multifunctional protease therapy of alkali burn-induced corneal ulceration, wherein corneal destruction is reduced or avoided. This example also demonstrates the benefit of the use of microgel alone which caused a reduction in corneal destruction, though not as markedly as when the microgel was applied in combination with the multifunctional protease.

Example 6.

This example sets forth methods for preparing hydrogel and microgel used in the context of the present invention. The microgel is used by itself or in combination with other agents, such as the krill-derived multifunctional hydrolases also set forth herein.

The chemicals and materials used therefor were: Glycerol (Merck, Darmstadt, GERMANY), Carbopol® polyanionic polymers (BFGoodrich Company, Specialty Polymers and Chemicals, Brecksville, OH), diisopropanol amine (Aldrich), distilled water, and 10% sodium hydroxide. The final concentrations of the component chemicals were: 23.5% w/v Glycerol stock (which is 87% w/w); 0.8% w/v of the desired polyanionic polymer; and distilled water and the sodium hydroxide (10%) or diisopropanol amine used to adjust the pH to 7.4 and make to volume.

Using standard sterile procedures, the carbopol was mixed in small amounts with distilled water under slow agitation with a propeller stirrer. The stirring continued until the powder was dissolved. Any trapped air was removed by reducing the pressure (water operated vacuum gauge). Glycerol was added under slow stirring and the pH was measured, and the 10% NaOH solution or the diisopropanol amine was used to adjust the composition to pH 7.4. Gelation occurred, resulting in a clear, transparent microgel. The resultant microgel was stored at 4°C.

Using the same methodology, but with weight to weight measurements of amounts, the following 10 g batches were made:

Batch 1	
Xanthan gum	0.6 g

Glycerol	2.058 g
sodium hydroxide pellets	quantity sufficient
sterile water	quantity sufficient
Batch 2	
Carbopol 934P	0.08 g
Glycerol	2.058 g
sodium hydroxide (10% w/w)	quantity sufficient
sterile water	quantity sufficient
Batch 3	
Carbopol 934P	0.04 g
Glycerol	2.058 g
40% w/w diisopropanolamine	quantity sufficient
sterile water	quantity sufficient
Batch 4	
Carbopol 971P	0.25 g
Glycerol	2.058 g
40% w/w diisopropanolamine	quantity sufficient
sterile water	quantity sufficient
Batch 5	
Carbopol 974P	0.08 g
glycerol	2.058 g
40% w/w diisopropanolamine	quantity sufficient
sterile water	quantity sufficient

* Keltrol-T brand, supplied by Monsanto,

Example 7.

The Krill broad-specificity serine protease activity was routinely assayed using Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma Chemical Co., St. Louis, MO) and the following chemicals, materials, and procedures:

The Assay Buffer is composed of 0.25 M HEPES (5.96 g HEPES/100 ml) and 1 mM $CaCl_2$ (100 µl 1 M $CaCl_2$ stock solution/100 ml). The solution is prepared by combining the HEPES and $CaCl_2$ in about 50 ml distilled water, adjusting the pH to 7.2 with 4 M NaOH and adding more distilled water to 100 ml. HEPES changes pH with temperature by -0.015 pH unit for each degree C. Therefore, the pH of the buffer is adjusted at the assay temperature (25°C).

The Substrate stock solution is 25 mM Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide dissolved in dimethylsulfoxide (DMSO) (156.2 mg/10 ml). The substrate does not dissolve well in DMSO that

contains water. Therefore the DMSO is stored in a container with desiccant. Dry DMSO becomes solid at 16-19°C.

The Enzyme solution is prepared by making dilutions thereof in distilled water. If the enzyme is stored on ice or in the refrigerator, the enzyme solution is allowed to equilibrate at room temperature for at least 10 minutes prior to the assay.

The assay was performed at 25°C in a thermostatted 1 ml polycarbonate cuvette. A change in the absorbance at 410 nm was monitored with time. The reaction rate in µmoles product formed per minute was calculated from the slope of the curve A410 *vs.* Time (min).

The assay was performed by placing via pipette the following into the cuvette: 920 µl Assay Buffer and 60 µl Substrate Stock Solution. The Assay Buffer-Substrate Stock Solution was stirred thoroughly back and forth with a cuvette stirrer to make sure that the substrate solution was completely mixed with the buffer (turning or swirling the cuvette stirrer in the solution was insufficient for thorough mixing). The stirrer was allowed to stand in the cuvette, which was placed in a rack in a waterbath at 25°C for at least 3 minutes. At the end of the incubation time, the cuvette was dried on the outside with a paper towel and placed in the thermostatted cuvette holder. Then 20 µl Enzyme solution was added to the cuvette using a liquid measuring device that delivers 20 µl with high accuracy (Hamilton syringe, P20 Gilson pipette, or low volume Rainin automatic pipette was used). All pipettes used were well calibrated: a 1 µl error in volume in this assay can result in a 5% error in the final result.

The Enzyme was mixed with the Substrate solution thoroughly with the cuvette stirrer (stir back and forth, not turn), the sample chamber was closed and the measurement was started by measuring the absorbance change with time over a 2 minute and 20 second period giving at least 6 slope readings over 20 seconds each. The first 20 seconds of each reaction is the lag time while the slopes of the next six 20 second periods are recorded. The two consecutive readings that give the highest slope in dA/min are chosen to represent the rate of the reaction. An average of those two numbers is calculated and multiplied by 5.68 to yield the activity concentration of the solution in U/ml.

The nucleic acid or amino acid sequences referred to herein by SEQ ID NOs: are as follows:

Example 8

The following example illustrates reduction of surgical adhesions by typical microgel formulations of the present invention. Compositions of the present invention were prepared as described in Example 6.

These compositions were tested for their ability to inhibit formation of adhesions in the rabbit sidewall model as follows.

Animals: Female New Zealand White rabbits, 2.4-2.7 kg, (Irish Farms, Norco, CA) were quarantined at least 2 days prior to use. The rabbits were housed on a 12:12 light:dark cycle with food and water available ad libitum.

Sidewall Model: Rabbits were anesthetized with a mixture of 55 mg/kg ketamine hydrochloride and 5 mg/kg Rompum intramuscularly. Following preparation for sterile surgery, a midline laparotomy

was performed. The cecum and bowel were exteriorized and digital pressure was exerted to create subserosal hemorrhages over all surfaces. The damaged intestine was then lightly abraded with 4", 4x4 ply sterile gauze until punctate bleeding was observed. The cecum and bowel were then returned to their normal anatomic position. A 3x5 cm area of peritoneum and transversus abdominous muscle was removed on the right lateral abdominal wall. Twelve mL of microgel without the enzymes of the present invention were distributed at the site of the injury. The incision was closed in two layers with 3-0 Dexon II. One week later, the animals were terminated and the percentage of the area of the sidewall injury that was involved in adhesions was determined. In addition, the tenacity of the adhesions was scored using the following system:

0 = No Adhesions

1 = mild, easily dissectable adhesions

2 = moderate adhesions; non-dissectable, does not tear the organ

3 = dense adhesions; non-dissectable, tears organ when removed

The results are given in Table 1 below. A reduction in either the frequency of occurrence, area, or tenacity of adhesions is considered to be beneficial. Each composition was tested on 10 rabbits.

TABLE 1

Composition	A	B	C	D	E	F[5]
Carbopol Used	971P	974P	934P	934P*	xanthan	none
Percent of Population Presenting Adhesions:	10	80	20	40	40	100
Mean Area of Adhesions Formed (%):	10	50	25	50	22.5	88
Mean Tenacity of Adhesions Formed:	1	1.3	1.5	1	1	2

* 934P* differs from 934P in the kind and amount of base used in the neutralization. 934P was neutralized with NaOH whilst 934P* was neutralized with di*iso*popanolamine.

[5] control

Example 9

The following example illustrates reduction of adhesions by typical microgel formulations of the present invention in which krill-derived multifunctional hydrolase of the present invention was included. Compositions of the present invention were prepared as described in Example 6, except that a sufficient amount of multifunctional krill-derived hydrolase, PHM-101, was added to the microgel formulation. Carbopol 974P was used in all formulations. The pH of the gel composition was adjusted to 7.4 as in previous examples. All of the other additives were also included in the placebo (blank) formulation.

The rabbit sidewall model experiments were carried out as described in example 8. The results are given in Table 2 below.

Group:	A	B	C	D	E	F*
Hydrolase Level (U/ml):	0	8.33	0.17	4.16	1.04	0
Percent of Population Presenting Adhesions:	60	70	33	70	50	100
Mean Area of Adhesions (%):	33	76	23	49	22	80
Mean Tenacity of Adhesions Formed:	1	1.3	1	1	1	1.9

* control, no enzyme or polyanionic polymer.

Example 10

The following example illustrates the reduction of adhesions by the krill-derived protease of the present invention.

The protease was administered in the form of an isotonic solution at a concentration of 0.2 U/ml/ Given that the solution was made up from a freeze-dried PHM-101 batch, it also contained the excipients described for the final manufacturing formula recited earlier in the application.

The rabbit sidewall model test was carried out as described in example 8 except that the preparation was delivered to the test site by miniosmotic pumps (Alza Corporation, Palo Alto, CA), which were tacked in place in a subcutaneous pocket by 4-0 Ethilon (Ethicon, Somerville, NJ). Pumps were replaced 3½ days, on average, after surgery. The results of the tests are given in Table 3 below.

TABLE 3

	Placebo	Protease
Percent of Population Presenting Adhesions:	90%	64%
Mean Areas of Adhesions (%):	64%	35%

Example 11

This example illustrates the clinical results obtained when a microgel formulation of the present invention containing a krill-derived protease of the present invention is used to treat a variety of ulcers, a form of wound, in humans.

The microgel formulation listed below was prepared by standard sterile formulary methods, analogous to those described in the previous examples. In all cases, Carbopol 974 was the hydrophilic polyanionic polymer. The krill-derived multifunctional hydrolase, PHM-101, lot CHX-088 was used. An

amount of hydrolase sufficient to bring the activity of the formulation to 0.2 U/ml. was added to the microgel formulation.

Component	Amount
Glycerol, 87% (Merk, Darmstadt, D)	23.5 g/liter soln'
Carbopol 974 (B.F. Goodrich, USA)	8.0 g/liter soln'
Redistilled Water	724 ml./liter soln'
10% $NaOH_{aq}$	sufficient to bring pH to 7.4±0.1

The results of several clinical trials carried-out with the above formulation are summarized in Table 4 below.

TABLE 4

Sex	Age	Diagnosis	Debridement	Odour	Pain	Healing
M	66	Decubitus ulcer	+			-
F	56	Lesions, locally advanced breast carcinoma	+			+
M	33	Decubitus ulcer	+			-
M	57	Decubitus ulcer	+			+
F	88	Venous ulcers of 20 years duration	+	+	+	+
M	74	Diabetic ulcer of leg	+	+	+	+
F	73	Arterial ulcer	+	+	+	+
M	70	Arterial ulcer	+	+	+	+
F	63	Decubitus ulcer	+	+	+	+
M	69	Arterial ulcer	+	+	+	+
F	64	Infected Surgical Wound (groin)	+	+	+	+

+ = A positive effect was observed where an effect was capable of observation

- = No effect was observed where an effect was capable of being seen.

Thus, 8 out of 10 patients exhibited an unexpected healing response to the treatment.

The foregoing examples serve to demonstrate the practice and usefulness of the present invention and in no way should they be construed as limiting the scope of the present invention.

The nucleic acid sequences described herein, and consequently the protein sequences derived therefrom, have been carefully sequenced. However, those of ordinary skill will recognize that nucleic acid sequencing technology can be susceptible to some inadvertent error. Those of ordinary skill in the relevant arts are capable of validating or correcting these sequences based on the ample description herein of methods of isolating the nucleic acid sequences in question, and such modifications that are made readily available by the present disclosure are encompassed by the present invention. Furthermore, those sequences reported herein are believed to define functional biological macromolecules within the invention whether or not later clarifying studies identify sequencing errors.

The application describes a number of nucleic acid sequences, of which the more germane are summarized below.

Short name	Nucleic Acid SEQ ID	Protein SEQ ID	Description
p62	1	2	Nucleic acid codes an LLLALVAAASAAEWRW sequence through to the end of the reading frame (as indicated by two in-frame stop codons).
p31	3	4	Nucleic acid codes a sequence that is extremely homologous to that beginning at AA117 of p62 through to the end of the reading frame.
p5.1a	5	6	Nucleic acid codes a sequence that is extremely homologous to AA1-170 of p62, plus an additional 7-amino acid sequence fused to the N-terminus.
p13	7	8	Nucleic acid encodes a sequence that is extremely homologous to AA73-283 of p62.
p912	9	10	Nucleic acid encodes a sequence that is extremely homologous to AA1-300 of p62, plus an additional 2-amino acid fused to the N-terminus.
p5.1b	11	12	Nucleic acid codes a sequence that is extremely homologous to AA1-170 of p62, plus an additional 7-amino acid sequence fused to the N-terminus. Comprises a consensus sequence derived from four PCR products.

While this invention has been described with an emphasis upon preferred embodiments, it will be obvious to those of ordinary skill in the art that variations in the preferred devices and methods may be used and that it is intended that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the claims that follow.

What is claimed is:

1. Use of a microgel comprising a crosslinked polyanionic polymer in the manufacture of a composition for treatment of an area affected by a trauma selected from the group consisting of corneal wounds and internal trauma.

2. The use of claim 1 wherein the corneal wound is a corneal ulcer, a corneal abrasion, or a chemical or physical insult to the cornea susceptible to giving rise to a corneal ulcer.

3. The use of claim 2 wherein the corneal ulcer is infected.

4. The use of claim 1 wherein the internal trauma is an internal surgical wound.

5. The use of claim 1 wherein the internal trauma comprises a trauma to a membrane that covers either an internal organ or tissue or the cavity in which one or more internal organs or tissues reside.

6. The use of claim 5 wherein the membrane is a serous membrane selected from the group consisting of the peritoneum, the pericardium, the epicardium, and the pleura.

7. The use of claim 5 wherein the membrane is an epithelium.

8. The use of claim 7 wherein the epithelium is the endothelium.

9. The use of claim 5 wherein the membrane is a meninges.

10. The use of claim 1 wherein the internal trauma is to a tendon or a tendon sheath.

11. The use of claim 1 wherein the internal trauma is to a nerve or a nerve sheath.

12. The use of claim 1 wherein the internal trauma is susceptible of giving rise to adhesions and the amount of microgel administered is an amount effective to prevent or reduce formation or reformation of adhesions.

13. The use of claim 1, wherein the composition further comprises a protein

14. The use of claim 13, wherein the protein is a protease whose activity comprising at least two of a chymotrypsin, trypsin, collagenase, and elastase activity.

15. The use of claim 14 wherein the protease is a multifunctional enzyme that is (a) a first enzyme and has at least about 60% sequence similarity with a reference sequence which is AA64-300 of SEQ ID NO:2 or AA1-300 of SEQ ID NO:2 or a sequence differing from these by at least one of the residue differences found in SEQ ID NO:4, 6, 8, 10, or 12 or (b) a second enzyme which is *Panaeus vanameii* 1, *Panaeus vanameii* 2, *Panaeus monodon* chymotryptic-1, *Panaeus monodon* tryptic, *Panaeus monodon* chymotryptic-2, *Uca pugilator* enzyme I, *Uca pugilator* enzyme II, Kamchatka crab IA, Kamchatka crab IIA, Kamchatka crab IIB, Kamchatka crab IIC, Crayfish protease 1, Salmon enzyme 1, Atlantic cod I, or Atlantic cod II.

16. The use of claim 15 wherein the wound is a corneal wound selected from the group consisting of a corneal ulcer, a corneal abrasion, and a chemical or physical insult to the cornea susceptible to giving rise to a corneal ulcer.

17. The use of claim 16 wherein the corneal ulcer is infected.

18. The use of claim 1 wherein the crosslinked polyanionic polymer is made by polymerization of one or more ethylenically unsaturated crosslinking agents and one or more ethylenically unsaturated compounds at least one of which has:

i) one or more functional groups that can be titrated with base to form negatively charged functional groups, or

ii) one or more precursor groups that are precursors of the functional groups that can be titrated with base; which precursor groups are converted to the functional groups.

19. The use of claim 18 wherein the functional groups are selected from $-C(O)OR^4$; $-S(O_2)OR^4$; or $-S(O)OR^4$; wherein R^4 is hydrogen, and wherein precursor groups are selected from $-C(O)R^4$, $-S(O_2)OR^4$, or $-S(O)OR^4$; wherein R^4 is independently C_1 - C_6 normal or branched alkyl, phenyl, or benzyl.

20. The use of claim 19 wherein the one or more ethylenically unsaturated compounds can be represented by the structure:

$(R^3)(R^2)C=C(R^1)$-X-Y

wherein:

Y is $-C(O)OR^4$; $-S(O_2)OR^4$; or $-S(O)OR^4$; wherein R^4 is hydrogen, C_1 to C_6 normal or branches alkyl, phenyl, or benzyl.

X is a direct bond; a straight or branched alkylene group having two to six carbon atoms, one or more of which can be replaced by O, S, or N heteroatoms, provided that there is no heteroatom in a position α or β to Y; phenylene; a five or six membered heteroarylene having up to three heteroatoms independently selected from O, S, and N, provided that neither Y or $R^3R^2C=C(R^1)$- is bonded to a heteroatom; and

R^1, R^2, and R^3 are independently selected from, hydrogen, C_1-C_6 alkyl, carboxy, halogen, cyano, isocyanato, C_1-C_6 hydroxyalkyl, alkoxyalkyl having 2 to 12 carbon atoms, C_1-C_6 haloalkyl, C_1-C_6 cyanoalkyl, C_3-C_6 cycloalkyl, C_1-C_6 carboxyalkyl, aryl, hydroxyaryl, haloaryl, cyanoaryl, C_1-C_6 alkoxyaryl, carboxyaryl, nitroaryl; and a group -X-Y; wherein C_1-C_6 alkyl or C_1-C_6 alkoxy groups are either linear or branched and up to Q-2 carbon atoms of any C_3-C_6 cycloalkyl group, wherein Q is the total number of ring carbon atoms in the cycloalkyl group, are independently replaced with O, S, or N heteroatoms; with the proviso that neither doubly-bonded carbon atom is directly bonded to O or S; and wherein aryl is phenyl or a 5 or 6 membered heteroaryl group having up to three heteroatoms selected from the group consisting of O, S, and N.

21. The use of claim 20 wherein R^1, R^2 and R^3 are independently hydrogen or C_1-C_3 alkyl and X is a direct bond or C_1-C_3 alkylene.

22. The use of claim 21 wherein R^2 and R^3 are H; R^1 is either hydrogen or methyl, and X is a direct bond.

23. The use of claim 22 wherein Y is $-C(O)OR^4$.

24. The use of claim 23 wherein R^4 is hydrogen or methyl.

25. The use of claim 24 wherein R^4 is hydrogen.

26. The use of claim 25 wherein the mole fraction of ethylenic double bonds in the combination from which the polyanionic polymer is made that is contributed by the ethylenically unsaturated crosslinking agent is 0.02 or less.

27. The use of claim 26 wherein the mole fraction of ethylenic double bonds in the mixture from which the crosslinked polyanionic polymer is made that is contributed by the ethylenically unsaturated crosslinking agent is 0.01 or less.

28. The use of claim 26 wherein the ethylenically unsaturated crosslinking agent is one of the triallylether of sucrose or the triallyl ether of pentaerythritol.

29. The use of claim 28 wherein the ethylenically unsaturated crosslinking agent is the triallyl ether of pentaerythritol.

30. The use of claim 1 wherein the ratio of the macroviscosity of the microgel to the microviscosity of the microgel is 10,000 or less.

31. Use of a microgel that comprises a crosslinked polyanionic polymer in the manufacture of a composition for reducing or preventing formation or reformation of adhesions in an area affected by a trauma susceptible to giving rise to adhesions an effective amount of.

32. Use of a microgel comprising crosslinked polyanionic polymer in the manufacture of a composition for treating the area affected by a wound wherein the crosslinked polyanionic polymer made by polymerization of one or more ethylenically unsaturated crosslinking agents and one or more ethylenically unsaturated compounds at least one of which has:

i) one or more functional groups that can be titrated with base to form negatively charged functional groups, or

ii) one or more precursor groups that are precursors of the functional groups that can be titrated with base; which precursor groups are converted to the functional groups: and wherein at least one of the following is true:

iii) the mole fraction of total ethylenic double bonds in the combination from which the crosslinked polyanionic polymer is made that is contributed by the ethylenically unsaturated crosslinking agent is 0.02 or less, or

iv) the ratio of macroviscosity of the microgel to the microviscosity of the microgel is 10,000 or less.

33. The use of claim 32 wherein the functional groups are selected from $-C(O)OR^4$; $-S(O_2)OR^4$; or $-S(O)OR^4$; wherein R^4 is hydrogen, and wherein precursor groups are selected from

$-C(O)R^4$, $-S(O_2)OR^4$, or $-S(O)OR^4$; wherein R^4 is independently C_1 - C_6 normal or branched alkyl, phenyl, or benzyl.

34. The use of claim 33 wherein at least one ethylenically unsaturated monomer can be represented by the structure

$$(R^3)(R^2)C=C(R^1)\text{-}X\text{-}Y$$

wherein:

Y is $-C(O)OR^4$; $-S(O_2)OR^4$; or $-S(O)OR^4$; wherein R^4 is hydrogen, C_1-C_6 alkyl, phenyl, or benzyl;

X is a direct bond; a straight or branched alkylene group having two to six carbon atoms, one or more of which can be replaced by O, S, or N heteroatoms, provided that there is no heteroatom in a position α or β to Y; phenylene; a five or six membered heteroarylene having up to three heteroatoms independently selected from O, S, and N, provided that neither Y or $R^3R^2C=C(R^1)$- is bonded to a heteroatom; and

R^1, R^2, and R^3 are independently selected from, hydrogen, C_1-C_6 alkyl, carboxy, halogen, cyano, isocyanato, C_1-C_6 hydroxyalkyl, alkoxyalkyl having 2 to 12 carbon atoms, C_1-C_6 haloalkyl, C_1-C_6 cyanoalkyl, C_3-C_6 cycloalkyl, C_1-C_6 carboxyalkyl, aryl, hydroxyaryl, haloaryl, cyanoaryl, C_1-C_6 alkoxyaryl, carboxyaryl, nitroaryl, and a group -X-Y; wherein C_1-C_6 alkyl or C_1-C_6 alkoxy groups are either linear or branched and up to Q-2 carbon atoms of any C_3-C_6 cycloalkyl group, wherein Q is the total number of ring carbon atoms in the cycloalkyl group, are independently replaced with O, S, or N heteroatoms; with the proviso that neither doubly-bonded carbon atom is directly bonded to O or S and wherein aryl is phenyl or a 5 or 6 membered heteroaryl group having up to three heteroatoms selected from the group consisting of O, S, and N.

35. The use of claim 34 wherein R^1, R^2, and R^3 are independently hydrogen or C_1-C_3 alkyl, X is a direct bond, and Y is $-C(0)OR^4$.

36. The use of claim 35 wherein R^1, R^2, and R^3 are hydrogen and R^4 is hydrogen.

37. The use of claim 32 wherein the composition further comprises a protein.

38. The use of a protein that has an activity comprising at least two of a chymotrypsin, trypsin, collagenase, and elastase activity in the manufacture of a composition for treating a corneal wound.

39. The use of claim 38 wherein the corneal wound is a corneal ulcer, a corneal abrasion, or a chemical or physical insult to the cornea susceptible to giving rise to a corneal ulcer.

40. The use of claim 39 wherein the corneal ulcer is infected.

41. The use of claim 38 wherein the protein is a multifunctional enzyme that is (a) a first enzyme and has at least about 60% sequence similarity with a reference sequence which is AA64-300 of SEQ ID NO:2 or AA1-300 of SEQ ID NO:2 or a sequence differing from these by at least one of the residue differences found in SEQ ID NO:4, 6, 8, 10, or 12 or (b) a second enzyme which is *Panaeus vanameii* **1**, *Panaeus vanameii* **2**, *Panaeus monodon* chymotryptic-1, *Panaeus monodon* tryptic, *Panaeus monodon* chymotryptic-2, *Uca pugilator* enzyme I, *Uca pugilator* enzyme II, Kamchatka crab IA, Kamchatka crab IIA, Kamchatka crab IIB, Kamchatka crab IIC, Crayfish protease 1, Salmon enzyme 1, Atlantic cod I, or Atlantic cod II.

42. Use of a microgel comprising a crosslinked polyanionic polymer in the manufacture of a composition for preventing or reducing the formation of adhesions following implantation of an implantable device wherein the crosslinked polyanionic polymer made by polymerization of one or more ethylenically unsaturated crosslinking agents and one or more ethylenically unsaturated compounds at least one of which has:

i) one or more functional groups that can be titrated with base to form negatively charged functional groups, or

ii) one or more precursor groups that are precursors of the functional groups that can be titrated with base: which precursor groups are converted to the functional groups.

43. The use of claim 42 wherein the functional groups are selected from $-C(O)OR^4$; $-S(O_2)OR^4$; or $-S(O)OR^4$; wherein R^4 is hydrogen, and wherein precursor groups are selected from $-C(O)R^4$, $-S(O_2)OR^4$, or $-S(O)OR^4$; wherein R^4 is independently C_1 - C_6 normal or branched alkyl, phenyl, or benzyl.

44. The use of claim 43 wherein the crosslinked polyanionic polymer is made from a combination of one or more ethylenically unsaturated crosslinking agents and one or more ethyleneically unsaturated compounds at least one of which can be represented by the structure

$$(R^3)(R^2)C=C(R^1)\text{-}X\text{-}Y$$

wherein:

Y is $-C(O)OR^4$; $-S(O_2)OR^4$; or $-S(O)OR^4$; wherein R^4 is hydrogen, C_1 to C_6 alkyl, phenyl, or benzyl.

X is a direct bond; a straight or branched alkylene group having two to six carbon atoms, one or more of which can be replaced by O, S, or N heteroatoms, provided that there is no

heteroatom in a position α or β to Y; phenylene; a five or six membered heteroarylene having up to three heteroatoms independently selected from O, S, and N, provided that neither Y or $R^3R^2C=C(R^1)$- is bonded to a heteroatom; and

R^1, R^2, and R^3 are independently selected from, hydrogen, C_1-C_6 alkyl, carboxy, halogen, cyano, isocyanato, C_1-C_6 hydroxyalkyl, alkoxyalkyl having 2 to 12 carbon atoms, C_1-C_6 haloalkyl, C_1-C_6 cyanoalkyl, C_3-C_6 cycloalkyl, C_1-C_6 carboxyalkyl, aryl, hydroxyaryl, haloaryl, cyanoaryl, C_1-C_6 alkoxyaryl, carboxyaryl, nitroaryl; and a group -X-Y; wherein C_1-C_6 alkyl or C_1-C_6 alkoxy groups are either linear or branched and up to Q-2 carbon atoms of any C_3-C_6 cycloalkyl group, wherein Q is the total number of ring carbon atoms in the cycloalkyl group, are independently replaced with O, S, or N heteroatoms; with the proviso that neither doubly-bonded carbon atom is directly bonded to O or S and wherein aryl is phenyl or a 5 or 6 membered heteroaryl group having up to three heteroatoms selected from the group consisting of O, S, and N.

45. The use of claim 42 wherein the microgel further comprises a protein.

46. The use of claim 42 wherein the mole fraction of ethylenic double bonds in the combination from which the polyanionic polymer is made contributed by the ethylenically unsaturated crosslinking agent is 0.02 or less.

47. The use of claim 42 wherein the ratio of the macroviscosity of the microgel to the microviscosity of the microgel is 10,000 or less.

48. A method of isolating a multifunctional proteolytic enzyme from a biological specimen comprising extracting the multifunctional proteolytic enzyme using fresh water.

49. The method of claim 48, wherein the biological specimen is not mechanically disrupted.

50. The method of claim 48, further comprising applying the fresh water extract to an affinity column having a ligand, wherein the ligand is aminophenylboronate.

51 A method of isolating a multifunctional proteolytic enzyme from a biological extract comprising applying the biological extract to an affinity column having a ligand, wherein the ligand is aminophenylboronate.

52 The method of claims 48 or 51 wherein the multifunctional proteolytic enzyme is (a) a first enzyme having activity comprising at least one of a chymotrypsin, trypsin, collagenase, and elastase activity, and at least about 60% sequence similarity with a reference sequence which is AA64-300 of SEQ ID NO:2 or AA!-300 of SEQ ID NO:2 or a sequence differing from these by at least one of the residue

differences found in SEQ ID NO:4, 6, 8, 10, or 12 or (b) a second enzyme which is *Panaeus vanamiii* 1, *Panaeus vanamiii* 2, *Panaeus monodon* chymotryptic-1, *Panaeus monodon* tryptic, *Panaeus monodon* chymotryptic-2, *Uca pugilator* enzyme I, *Uca pugilator enzyme* II, Kamchatka crab IA, Kamchatka crab IIA, Kamchatka crab IIB, Kamchatka crab IIC Crayfish protease 1, Salmon enzyme 1, Atlantic cod I, or Atlantic cod II.

Nucleotide alignment of p62 with p31

88.0% identity in 590 bp overlap

```
          320       330       340       350       360       370
p62  TATGGATGGTGCTGGGTTTGTTGAGGTTGTGATGGGTGCTCACAGTATCCATGACGAAAC
                                       ||||||||||||||||||||||||||| ||
p31                                    GATGGGTGCTCACAGTATCCATGACGATAC
                                              10        20        30

          380       390       400       410       420       430
p62  TGAGGCCACACAGGTCCGTGCCACATCAACTGATTTCTTCACCCACGAGAACTGGAACTC
     ||||||| | |  ||| ||||||||||||||||||||||||||||||||||||||||||||
p31  TGAGGCCTCTCGCGTCAGTGCCACATCAACTGATTTCTTCACCCACGAGAACTGGAACTC
               40        50        60        70        80        90

          440       450       460       470       480       490
p62  CTTCACCCTCTCCAATGATCTTGCTCTCATTAAGATGCCAGCACCAATTGAATTCAACGA
     |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
p31  CTTCACCCTCACCAATGATCTTGCTCTCATTAAGATGCCAGCACCAATTGAATTCACACC
              100       110       120       130       140       150

          500       510       520       530       540       550
p61  TGTGATCCAGCCTGTCTGCCTACCAACCTATACTGATGCTAGTGATGATTTTGTTGGTGA
     ||  || ||  ||||||||||||||||  ||| |||||||||  |||||||||  |||||||
p31  TGAAATTCAACCTGTCTGCCTACCAAGCTACACTGATGCTGCTGATGATTTCATTGGTGA
              160       170       180       190       200       210

          560       570       580       590       600       610
p62  ATCAGTCACTCTTACTGGATGGGGTAAACCATCTGACTCTGCTTTTGGCATCGCTGAACA
     |||  ||    ||||||||||||||      |||||  ||||||  ||||||  ||||||
p31  ATCTGTTGTCCTTACTGGATGGGGCCGTGATTCTGATGCTGCTTCCGGCATCTCTGAACT
              220       230       240       250       260       270

          620       630       640       650       660       670
p62  ACTTCGTGAGGTTGATGTGACAACAATCACTACTGCTGACTGCCAGGCATACTACGGCAT
     |||  |||||||||  |||||||  ||||||  |  |||||  ||||||||||||||||||||
p31  ACTCCGTGAGGTTCATGTGACCACAATCTCCACTGCCGACTGCCAGGCATACTACGGCAT
              280       290       300       310       320       330

          680       690       700       710       720       730
p62  TGTCACTGACAAAATCCTCTGCATCGACTCCGAAGGAGGCCATGGTTCCTGCAATGGTGA
     |||||||||||||||||||||||||    |||  ||||   ||  ||||||||  ||  ||||||||
p31  TGTCACTGACAAAATCCTCTGCATTTCCTCTGAAGACGGACATGGTTCTTGTAATGGTGA
              340       350       360       370       380       390

          740       750       760       770       780       790
p62  TTCCGGCGGGCCAATGAACTATGTAACTGGTGGTGTTACTCAGACCCGTGGTATTACCTC
     ||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||
p31  TTCCGGTGGGCCAATGAACTATGTAACTGGTGGTGTTACTCAGACCCGTGGTATTACCTC
              400       410       420       430       440       450

          800       810       820       830       840       850
p62  TTTCGGATCCTCTACCGGCTGCGAGACTGGCTACCCTGATGGTTACACACGAGTCACCAG
     ||||||||||||||||  ||  ||||||||||||||||||||||||||||  ||||||||
p31  CTTCGGATCCTCTACCGGGTGTGAGACTGGCTACCCTGATGGTTACACACGTGTCACCAG
              460       470       480       490       500       510

          860       870       880       890       900       910
p62  CTATCTGGACTGGATTGAATCTAACACTGGCATTGCCATTGATCCATAAATACAATTCTA
     ||||||||||||||||||||||||||||||||||||||||  |  |  ||||  |||  |||
p31  CTATCTGGACTGGATTGAATCTAACACTGGCATTGCCATTGATGCTTGAATATAATACTA
              520       530       540       550       560       570

          920       930       940
p62  GCAA--AAATACAATAAATTATACTTAAATG
     |  |   |||  ||||||||
p31  GATATGTAATCAAATAAATTTCATGAATT
              580       590
```

Figure 1

Protein Alignment of p62 and p11

~89% identity in 189 amino acid overlap

```
p62                                                    LLLALVAAASAAEWRWQFRHPTVTP

p62     NPRAKNPFRVTKSSPVQPPAVRGTKAVENCGPVAPRNKIVGGMEVTPHAYPWQVGLFIDD

p62     MYFCGGSIISDEWVLTAAHCMDGAGFVEVVMGAHSIHDETEATQVRATSTDFFTHENWNS
                                        ||||||||:|||::|:|||||||||||||||
p11                                     MGAHSIHDDTEASRVSATSTDFFTHENWNS

p62     FTLSNDLALIKMPAPIEFNDVIQPVCLPTYTDASDDFVGESVTLTGWGKPSDSAFGIAEQ
        |||:|||||||||||||:  |||||||:||||:|||:||||:||||||: ||:| ||:|
p11     FTLTNDLALIKMPAPIEFTPEIQPVCLPSYTDAADDFIGESVVLTGWGRDSDAASGISEL

p62     LREVDVTTITTADCQAYYGIVTDKILCIDSEGGHGSCNGDSGGPMNYVTGGVTQTRGITS
        ||||:|||||:|||||||||||||||||||:||:|||||||||||||||||||||||||||
p11     LREVHVTTISTADCQAYYGIVTDKILCISSEDGHGSCNGDSGGPMNYVTGGVTQTRGITS

p62     FGSSTGCETGYPDGYTRVTSYLDWIESNTGIAIDP
        |||||||||||||||||||||||||||||||||||:
p11     FGSSTGCETGYPDGYTRVTSYLDWIESNTGIAIDA
```

Figure 2

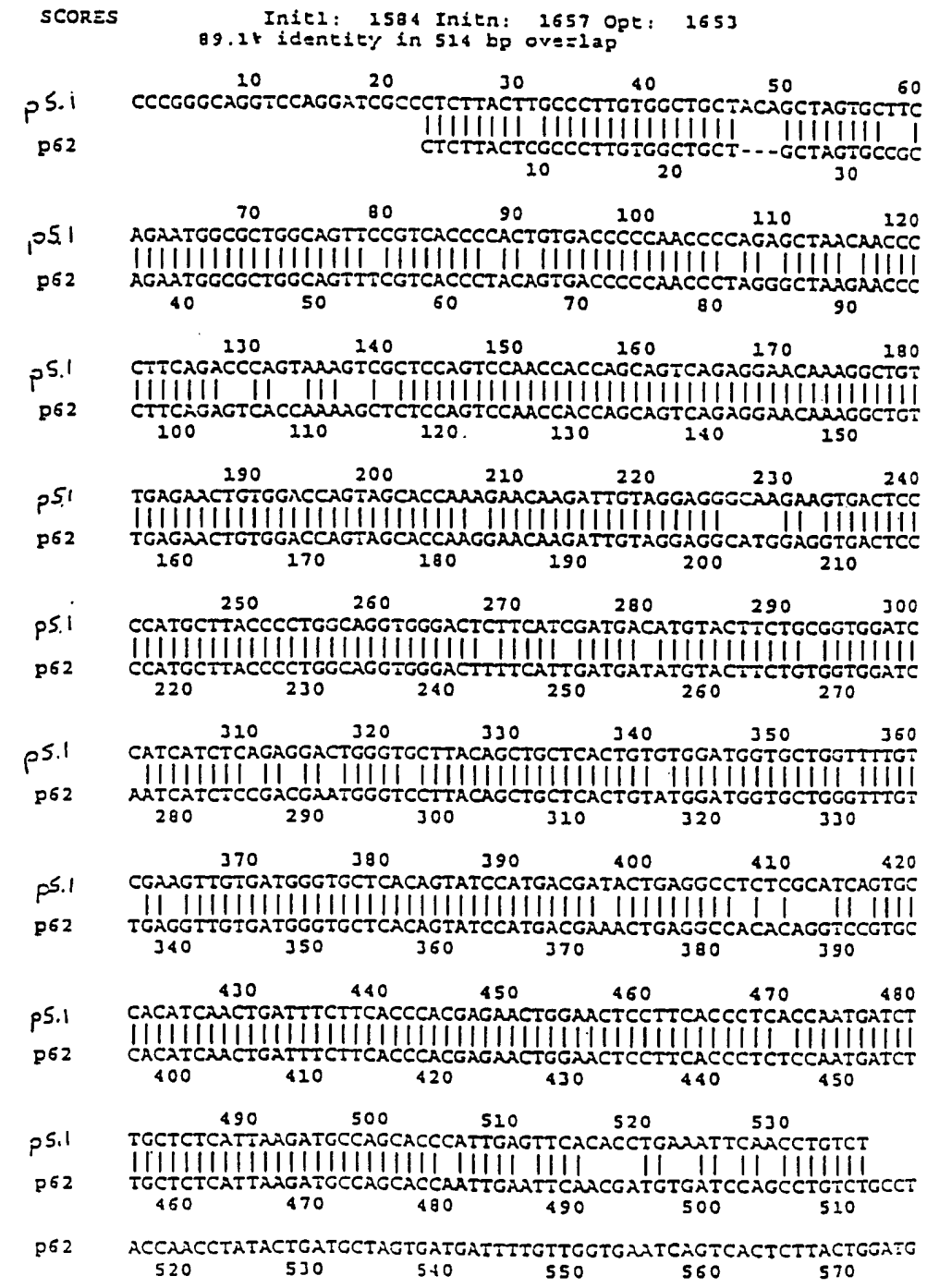
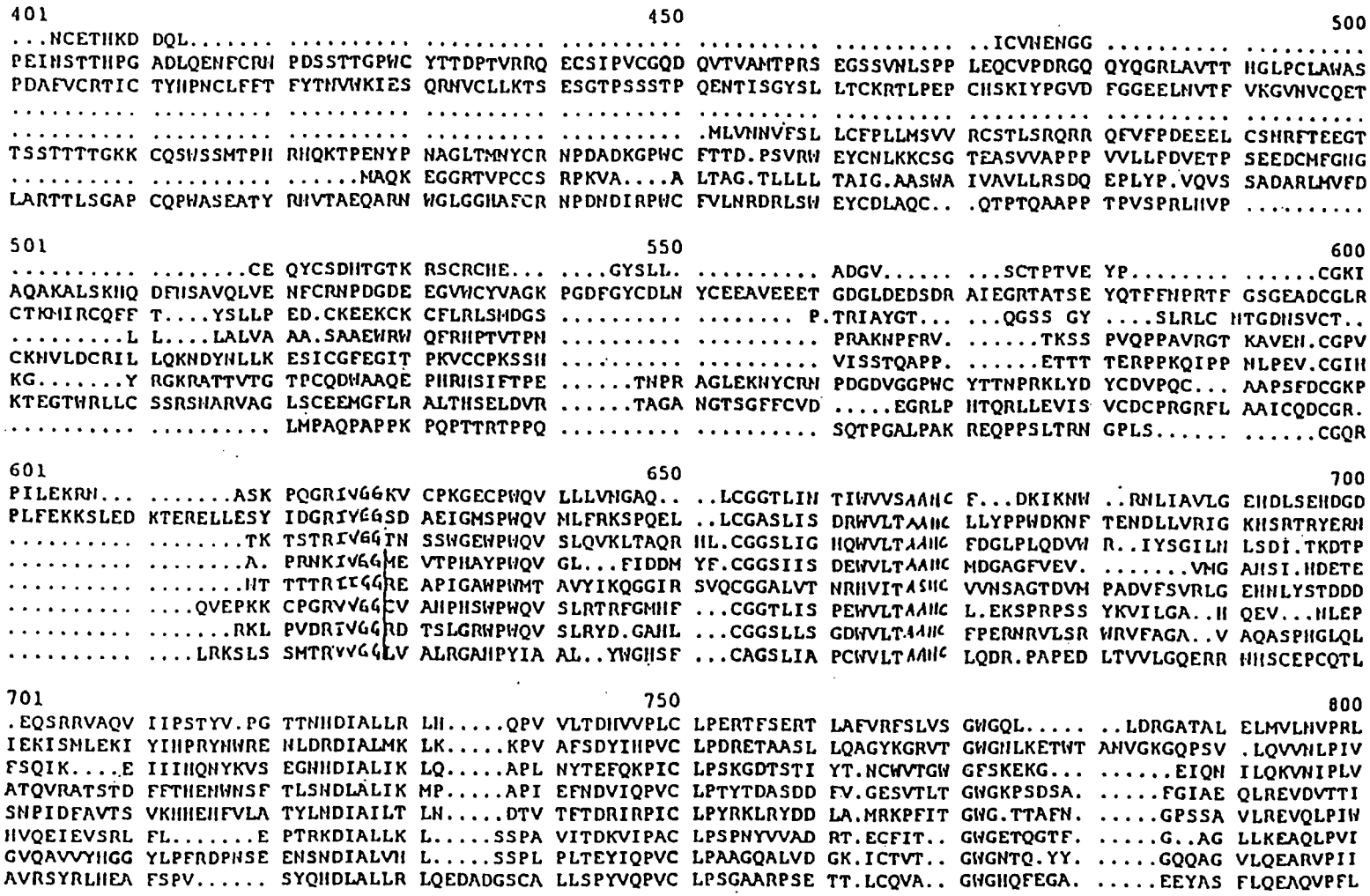

SCORES Initl: 1584 Initn: 1657 Opt: 1653
89.1% identity in 514 bp overlap
pS.1
p62

Figure 3

```
          1                                                   50
p5.1      PGRSRIALLL ALVAATASAS EWRWQFRHPT VTPNPRANNP FRPSKVAPVQ
p62       .......LLL ALVAA.ASAA EWRWQFRHPT VTPNPRAKNP FRVTKSSPVQ
p31       .......... .......... .......... .......... ..........

          51                                                  100
p5.1      PPAVRGTKAV ENCGPVAPKN KIVGGQEVTP HAYPWQVGLF IDDMYFCGGS
p62       PPAVRGTKAV ENCGPVAPRN KIVGGMEVTP HAYPWQVGLF IDDMYFCGGS
p31       .......... .......... .......... .......... ..........

          101                                                 150
p5.1      IISEDWVLTA AHCVDGAGFV EVVMGAHSIH D TEA  I A TSTDFFTHEN
p62       IISDEWVLTA AHCMDGAGFV EVVMGAHSIH DETEATQVRA TSTDFFTHEN
p31       .......... .......... ...MGAHSIH D TEA  V A TSTDFFTHEN

          151                                                 200
p5.1      WNSFTL NDL ALIKMPAPIE F   IQPV.. .......... ..........
p62       WNSFTLSNDL ALIKMPAPIE FNDVIQPVCL PTYTDASDDF VGESVTLTGW
p31       WNSFTL NDL ALIKMPAPIE F   IQPVCL PSYTDAADDF IGESVVLTGW

          201                                                 250
p5.1      .......... .......... .......... .......... ..........
p62       GKPSDSAFGI AEQLREVDVT TITTADCQAY YGIVTDKILC IDSEGGHGSC
p31       GRDSDAASGI SELLREVHVT TISTADCQAY YGIVTDKILC ISSEDGHGSC

          251                                                 300
p5.1      .......... .......... .......... .......... ..........
p62       NGDSGGPMNY VTGGVTQTRG ITSFGSSTGC ETGYPDGYTR VTSYLDWIES
p31       NGDSGGPMNY VTGGVTQTRG ITSFGSSTGC ETGYPDGYTR VTSYLDWIES

          301                 322
p5.1      .......... .......... ..
p62       NTGIAIDP.. .......... ..
p31       NTGIAIDA
```

Figure 4


401 450 500
F VII ...NCETHKD DQL..... ....ICVHENGG ....
hrombin PEINSTTHPG ADLQENFCRN PDSSTTGPWC YTTDPTVRRQ ECSIPVCGQD QVTVAMTPRS EGSSVNLSPP LEQCVPDRGQ QYQGRLAVTT HGLPCLAWAS
likrein PDAFVCRTIC TYHPNCLFFT FYTNVWKIES QRNVCLLKTS ESGTPSSSTP QENTISGYSL LTCKRTLPEP CHSKIYPGVD FGGEELNVTF VKGVNVCQET
p62 ....
Limulus .... .MLVNNVFSL LCFPLLMSVV RCSTLSRQRR QFVFPDEEEL CSNRFTEEGT
Plasmin TSSTTTTGKK CQSWSSMTPH RHQKTPENYP NAGLTMNYCR NPDADKGPWC FTTD.PSVRW EYCNLKKCSG TEASVVAPPP VVLLPDVETP SEEDCMFGHG
Hepsin ....NAQK EGGRTVPCCS RPKVA....A LTAG.TLLLL TAIG.AASWA IVAVLLRSDQ EPLYP.VQVS SADARLHVFD
F XII LARTTLSGAP CQPWASEATY RNVTAEQARN WGLGGHAFCR NPDNDIRPWC FVLNRDRLSW EYCDLAQC.. .QTPTQAAPP TPVSPRLHVP .....

501 550 600
F VII ....CE QYCSDHTGTK RSCRCHE... ....GYSLL. ..... ADGV..... ...SCTPTVE YP..... .....CGKI
hrombin AQAKALSKHQ DFHSAVQLVE NFCRNPDGDE EGVWCYVAGK PGDFGYCDLN YCEEAVEEET GDGLDEDSDR AIEGRTATSE YQTFFNPRTF GSGEADCGLR
likrein CTKNIRCQFF T....YSLLP ED.CKEEKCK CFLRLSHDGS .... P.TRIAYGT... ...QGSS GY ....SLRLC NTGDHSVCT..
p62 .....L L....LALVA AA.SAAEWRW QFRHPTVTPN .... PRAKNPFRV. .....TKSS PVQPPAVRGT KAVEN.CGPV
Limulus CKNVLDCRIL LQKNDYNLLK ESICGFEGIT PKVCCPKSSH .... VISSTQAPP. .....ETTT TERPPKQIPP NLPEV.CGIH
Plasmin KG.....Y RGKRATTVTG TPCQDWAAQE PHRHSIFTPE .....TNPR AGLEKNYCRN PDGDVGGPWC YTTNPRKLYD YCDVPQC... AAPSFDCGKP
Hepsin KTEGTWRLLC SSRSHARVAG LSCEEMGFLR ALTHSELDVR .....TAGA NGTSGFFCVD .....EGRLP HTQRLLEVIS VCDCPRGRFL AAICQDCGR.
F XII .... LMPAQPAPPK PQPTTRTPPQ .... SQTPGALPAK REQPPSLTRN GPLS..... .....CGQR

601 650 700
F VII PILEKRN... .....ASK PQGRIVGGKV CPKGECPWQV LLLVNGAQ.. ..LCGGTLIN TIWVVSAAHC F...DKIKNW ..RNLIAVLG EHDLSEHDGD
hrombin PLFEKKSLED KTERELLESY IDGRIYEGSD AEIGMSPWQV MLFRKSPQEL ..LCGASLIS DRWVLTAAHC LLYPPWDKNF TENDLLVRIG KHSRTRYERN
likrein ....TK TSTRIVGGTN SSWGEWPWQV SLQVKLTAQR HL.CGGSLIG HQWVLTAAHC FDGLPLQDVW R..IYSGILN LSDI.TKDTP
p62 ....A. PRNKIVGGME VTPHAYPWQV GL...FIDDM YF.CGGSIIS DEWVLTAAHC MDGAGFVEV. .....VMG AHSI.HDETE
Limulus ....HT TTTRIIGGRE APIGAWPWMT AVYIKQGGIR SVQCGGALVT NRHVITAAHC VVNSAGTDVM PADVFSVRLG EHNLYSTDDD
Plasmin ....QVEPKK CPGRVVGGCV AHPHSWPWQV SLRTRFGMHF ...CGGTLIS PEWVLTAAHC L.EKSPRPSS YKVILGA..H QEV...NLEP
Hepsin ....RKL PVDRIVGGRD TSLGRWPWQV SLRYD.GAHL ...CGGSLLS GDWVLTAAHC FPERNRVLSR WRVFAGA..V AQASPHGLQL
F XII ....LRKSLS SMTRVVGGLV ALRGAHPYIA AL..YWGHSF ...CAGSLIA PCWVLTAAHC LQDR.PAPED LTVVLGQERR HHSCEPCQTL

701 750 800
F VII .EQSRRVAQV IIPSTYV.PG TTNHDIALLR LH.....QPV VLTDHVVPLC LPERTFSERT LAFVRFSLVS GWGQL..... ..LDRGATAL ELMVLNVPRL
hrombin IEKISMLEKI YIHPRYNWRE NLDRDIALMK LK.....KPV AFSDYIHPVC LPDRETAASL LQAGYKGRVT GWGNLKETWT ANVGKGQPSV .LQVVNLPIV
likrein FSQIK....E IIIHQNYKVS EGNHDIALIK LQ.....APL NYTEFQKPIC LPSKGDTSTI YT.NCWVTGW GFSKEKG... .....EIQN ILQKVNIPLV
p62 ATQVRATSTD FFTHENWNSF TLSNDLALIK MP.....API EFNDVIQPVC LPTYTDASDD FV.GESVTLT GWGKPSDSA. .....FGIAE QLREVDVTTI
Limulus SNPIDFAVTS VKHHEHFVLA TYLNDIAILT LN.....DTV TFTDRIRPIC LPYRKLRYDD LA.MRKPFIT GWG.TTAFN. .....GPSSA VLREVQLPIW
Plasmin HVQEIEVSRL FL.....E PTRKDIALLK L.....SSPA VITDKVIPAC LPSPNYVVAD RT.ECFIT.. GWGETQGTF. .....G..AG LLKEAQLPVI
Hepsin GVQAVVYHGG YLPFRDPHSE ENSNDIALVH L.....SSPL PLTEYIQPVC LPAAGQALVD GK.ICTVT.. GWGNTQ.YY. .....GQQAG VLQEARVPII
F XII AVRSYRLHEA FSPV..... SYQHDLALLR LQEDADGSCA LLSPYVQPVC LPSGAARPSE TT.LCQVA.. GWGHQFEGA. .....EEYAS FLQEAQVPFL

Figure 5

```
          801                                                  850                                                  900
 F VII    MTQDCLQQSR KVGDSPNITE YMFCAGY.SD GSK..DSCKG DSGGP|HA..T HY..RGTWYL TGIVSWGQGC ATVGHF.GVY TRVSQYIEWL QKLMRSEPRP
rombin    ERPVC..... KDSTRIRITD NMFCAGYKPD EGKRGDACEG DSGGPFVMKS PF..NNRWYQ MGIVSWGEGC DRDGKY.GFY THVFRLKKWI QKVIDQFGE.
ikrein    TNEECQKRYQ .DY...KITQ RMVCAGYKEG GK...DACKG DSGGPLVC..  ..KHNGMWRL VGITSWGEGC ARREQ.PGVY TKVAEYMDWI LEKTQSSDGK
   p62    TTADCQAYYG ......IVTD KILCID.SEG GH...GSCNG DSGGPMNY..  ..VTGGVTQT RGITSFGSST GCETGYPDGY TRVTSYLDWI ESNTGIAIDP
imulus    EHEACRQAYE KDL...NITN VYMCAGFADG GK...DACQG DSGGPMML..  .PVKTGEFYL IGIVSFGKKC A.LPGFPGVY TKVTEFLDWI AEHMV.....
lasmin    ENKVCHRYEF LNG...RVQS TELCAGHLAG GT...DSCQG DSGGPLVCFE  ....KDKYIL QGVTSWGLGC A.RPNKPGVY VRVSRFVTWI EGVMRNN...
Hepsin    SNDVCNGADF YGN...QIKP KMFCAGYPEG GI...DACQG DSGGPFVCED SISRTPRWRL CGIVSWGTGC A.LAQKPGVY TKVSDFREWI FQAIKTHSEA
 F XII    SLERCSAPDV HGS...SILP GMLCAGFLEG GT...DACQG DSGGPLVCED QAA.ERRLTL QGIISWGSGC G.DRNKPGVY TDVAYYLAWI REHTVS....

          901
 F VII    GVLLRAPFP
rombin    .........
ikrein    AQMQSPA..
   p62    .........
imulus    .........
lasmin    .........
Hepsin    SGMVTQL..
 F XII    .........
```

Figure 5, Continued

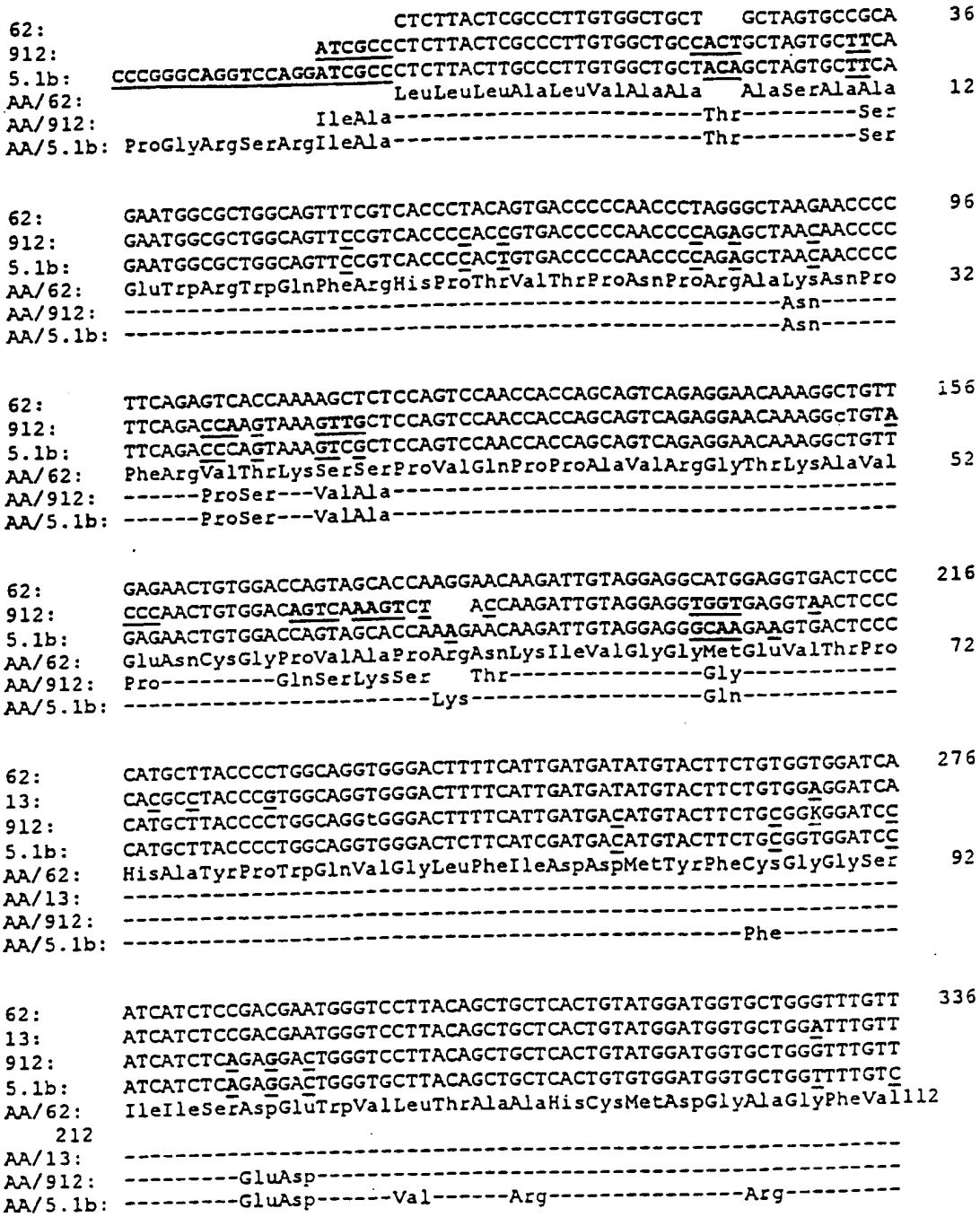


Figure 6 - Part 1

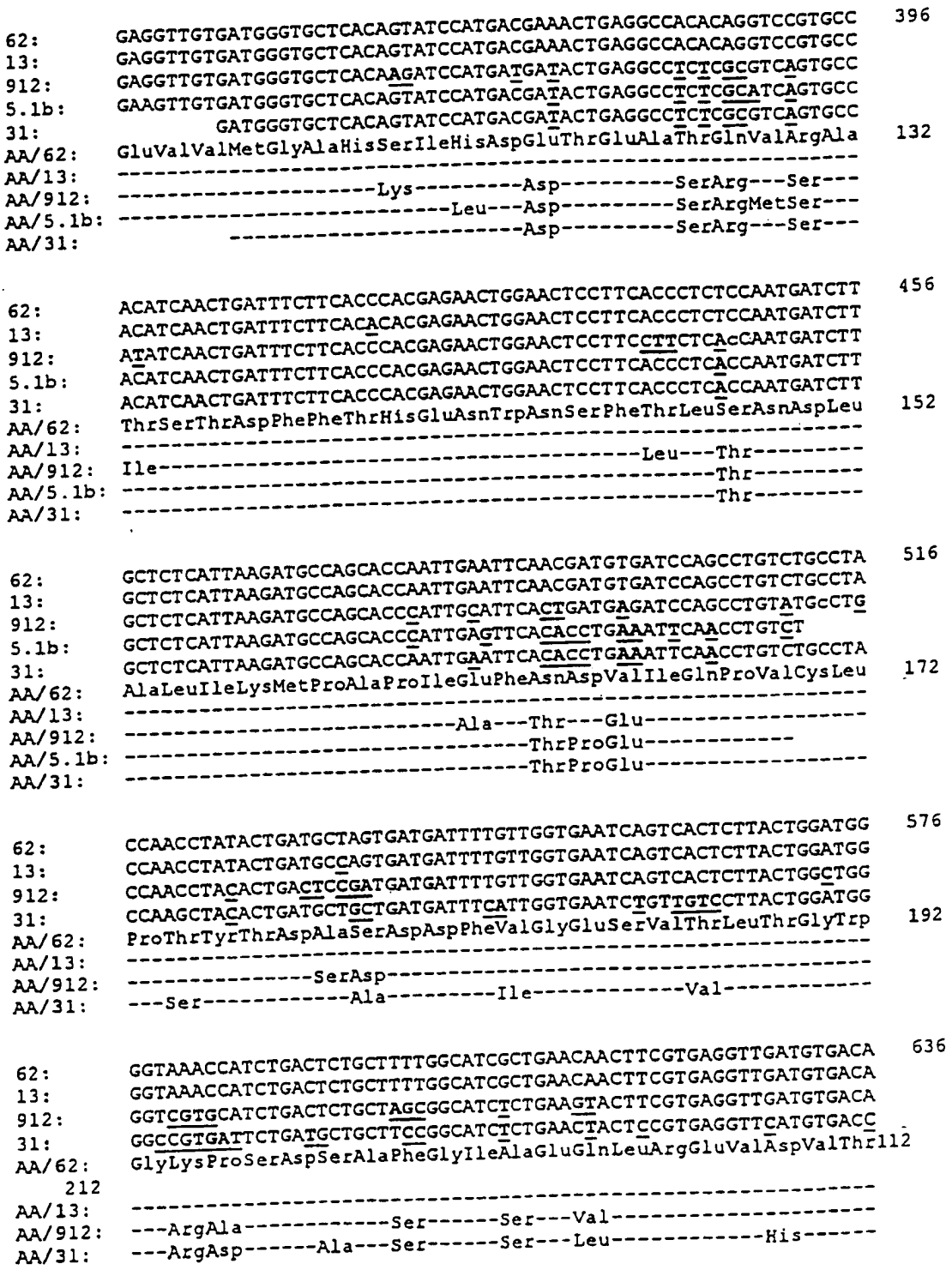


Figure 6 - Part 2

```
62:       Acaatcactactgctgactgccaggcatactacggcattgtcactgacaaaatcctctgc   696
13:       ACaatcactactgctgactgccaggcatactacggcattgtcactgacaaaatcctctgc
912:      ACAATAACTACTGCCGACTGCCAGGCATACTATGGTATTGTCACTGACAAAATCCTCTGC
31:       ACAATCTCCACTGCCGACTGCCAGGCATACTACGGCATTGTCACTGACAAAATCCTCTGC
AA/62:    ThrIleThrThrAlaAspCysGlnAlaTyrTyrGlyIleValThrAspLysIleLeuCys132
    232
AA/13:    ------------------------------------------------------------
AA/912:   ------------------------------------------------------------
AA/31:    ------Ser-------------------------------------------------

62:       atcgactccgaaggaggccatggttcctgcaatggtgattccggCgggccaatgaactat   756
13:       atcgactccgaaggaggccatggttcctgcaatggtgattccggtgggccaatgaactat
912:      ATCGACTCAGAAGGAGGTCATGGGTCTTGCAATGGTGATTCCGGTGGGCCAATGAACTAT
31:       ATTTCCTCTGAAGACGGACATGGTTCTTGTAATGGTGATTCCGGTGGGCCAATGAACTAT
AA/62:    IleAspSerGluGlyGlyHisGlySerCysAsnGlyAspSerGlyGlyProMetAsnTyr152
    252
AA/13:    ------------------------------------------------------------
AA/912:   ------------------------------------------------------------
AA/31:    ---Ser------Asp-------------------------------------------

62:       gtaactggtggtgttactcagacccgtggtattacctctttcggatcctctaccggctgc   816
13:       gtaactggtggtgttactcagacccgtggtattacctctttcggatcctctaccggctgc
912:      GTAACTGGTGGTGTTACTCAGACCCGTGGTATTACCtcCTTCGGATCCTCTACCGGCTGT
31:       GTAACTGGTGGTGTTACTCAGACCCGTGGTATTACCTCCTTCGGATCCTCTACCGGGTGT
AA/62:    ValThrGlyGlyValThrGlnThrArgGlyIleThrSerPheGlySerSerThrGlyCys172
    272
AA/13:    ------------------------------------------------------------
AA/912:   ------------------------------------------------------------
AA/31:    ------------------------------------------------------------

62:       gagactggCtaccctgatGGttacacacgagtcACCAGCTATCTGGACTGGATTGAATCT   876
13:       gagactgggtaccctgataattacacacgagtc
912:      GAGACTGGCTACCCTGATGGTTACACACGAGTCACCAGCTATCTAGACTGGATTGAATCT
31:       GAGACTGGCTACCCTGATGGTTACACACGTGTCACCAGCTATCTGGACTGGATTGAATCT
AA/62:    GluThrGlyTyrProAspGlyTyrThrArgValThrSerTyrLeuAspTrpIleGluSer192
    292
AA/13:    ------------------Asn-------------
AA/912:   ------------------------------------------------------------
AA/31:    ------------------------------------------------------------

62:       AACACTGGCATTGCCATTGATCCATAAATACAATTCTAGCAAAAATACAATAAATTATAC   936
912:      AACACTGGCATTGCCATTGATCCTTGAATAATATTCTAGCTGAATGATAATAAATTCATG
31:       AACACTGGCATTGCCATTGATGCTTGAATATAATACTAGATATGTAATCAAATAAATTTC
AA/62:    AsnThrGlyIleAlaIleAspPro*                                      300
AA/912:   ------------------------*
AA/31:    ----------------------Ala*

62:       TTAAATG                                                        943
912:      ATTGATAATCAAAAAAAAAAAAAAA
31:       ATGAATT
```

Figure 6 - Continued - Part 3

```
62:                                 L  L  L  A  L  V  A  A     A  S  A  A   12
912:                          I  A  L  L  L  A  L  V  A  A  T  A  S  A  S   15
5.1b:  P  G  R  S  R  I  A  L  L  L  A  L  V  A  A  T  A  S  A  S   20

62:    E  W  R  W  Q  F  R  H  P  T  V  T  P  N  P  R  A  K  N  P   32
912:   E  W  R  W  Q  F  R  H  P  T  V  T  P  N  P  R  A  N  N  P   35
5.1b:  E  W  R  W  Q  F  R  H  P  T  V  T  P  N  P  R  A  N  N  P   40

62:    F  R  V  T  K  S  S  P  V  Q  P  P  A  V  R  G  T  K  A  V   52
912:   F  R  P  S  K  V  A  P  V  Q  P  P  A  V  R  G  T  K  A  V   55
5.1b:  F  R  P  S  K  V  A  P  V  Q  P  P  A  V  R  G  T  K  A  V   60

62:    E  N  C  G  P  V  A  P  R  N  K  I  V  G  G  M  E  V  T  P   72
912:   P  N  C  G  Q  S  K  S     T  K  I  V  G  G  G  E  V  T  P   74
5.1b:  E  N  C  G  P  V  A  P  K  N  K  I  V  G  G  Q  E  V  T  P   80

62:    H  A  Y  P  W  Q  V  G  L  F  I  D  D  M  Y  F  C  G  G  S   92
13:    H  A  Y  P  W  Q  V  G  L  F  I  D  D  M  Y  F  C  G  G  S   20
912:   H  A  Y  P  W  Q  V  G  L  F  I  D  D  M  Y  F  C  G  G  S   94
5.1b:  H  A  Y  P  W  Q  V  G  L  F  I  D  D  M  Y  F  F  G  G  S  100

62:    I  I  S  D  E  W  V  L  T  A  A  H  C  M  D  G  A  G  F  V  112
13:    I  I  S  D  E  W  V  L  T  A  A  H  C  M  D  G  A  G  F  V   40
912:   I  I  S  E  D  W  V  L  T  A  A  H  C  M  D  G  A  G  F  V  114
5.1b:  I  I  S  E  D  W  V  V  T  A  R  H  C  M  D  G  R  G  F  V  120

62:    E  V  V  M  G  A  H  S  I  H  D  E  T  E  A  T  Q  V  R  A  132
13:    E  V  V  M  G  A  H  S  I  H  D  E  T  E  A  T  Q  V  R  A   60
912:   E  V  V  M  G  A  H  K  I  H  D  D  T  E  A  S  R  V  S  A  134
5.1b:  E  V  V  M  G  A  H  S  I  L  D  D  T  E  A  S  R  M  S  A  140
31:                   M  G  A  H  S  I  H  D  D  T  E  A  S  R  V  S  A   17

62:    T  S  T  D  F  F  T  H  E  N  W  N  S  F  T  L  S  N  D  L  152
13:    T  S  T  D  F  F  T  H  E  N  W  N  S  F  T  L  S  N  D  L   80
912:   I  S  T  D  F  F  T  H  E  N  W  N  S  F  L  L  T  N  D  L  154
5.1b:  T  S  T  D  F  F  T  H  E  N  W  N  S  F  T  L  T  N  D  L  160
31:    T  S  T  D  F  F  T  H  E  N  W  N  S  F  T  L  T  N  D  L   37

62:    A  L  I  K  M  P  A  P  I  E  F  N  D  V  I  Q  P  V  C  L  172
13:    A  L  I  K  M  P  A  P  I  E  F  N  D  V  I  Q  P  V  C  L  100
912:   A  L  I  K  M  P  A  P  I  A  F  T  D  E  I  Q  P  V  C  L  174
5.1b:  A  L  I  K  M  P  A  P  I  E  F  T  P  E  I  Q  P  V        180
31:    A  L  I  K  M  P  A  P  I  E  F  T  P  E  I  Q  P  V  C  L   57

62:    P  T  Y  T  D  A  S  D  D  F  V  G  E  S  V  T  L  T  G  W  192
13:    P  T  Y  T  D  A  S  D  D  F  V  G  E  S  V  T  L  T  G  W  120
912:   P  T  Y  T  D  S  D  D  D  F  V  G  E  S  V  T  L  T  G  W  194
31:    P  S  Y  T  D  A  A  D  D  F  I  G  E  S  V  V  L  T  G  W   77

62:    G  K  P  S  D  S  A  F  G  I  A  E  Q  L  R  E  V  D  V  T  212
13:    G  K  P  S  D  S  A  F  G  I  A  E  Q  L  R  E  V  D  V  T  140
912:   G  R  A  S  D  S  A  S  G  I  S  E  V  L  R  E  V  D  V  T  214
31:    G  R  D  S  D  A  A  S  G  I  S  E  L  L  R  E  V  H  V  T   97
```

Figure 7 - Part 1

```
62:    T I T T A D C Q A Y Y G I V T D K I L C   232
13:    T I T T A D C Q A Y Y G I V T D K I L C   160
912:   T I T T A D C Q A Y Y G I V T D K I L C   234
31:    T I S T A D C Q A Y Y G I V T D K I L C   117

62:    I D S E G G H G S C N G D S G G P M N Y   252
13:    I D S E G G H G S C N G D S G G P M N Y   180
912:   I D S E G G H G S C N G D S G G P M N Y   254
31:    I S S E D G H G S C N G D S G G P M N Y   137

62:    V T G G V T Q T R G I T S F G S S T G C   272
13:    V T G G V T Q T R G I T S F G S S T G C   200
912:   V T G G V T Q T R G I T S F G S S T G C   274
31:    V T G G V T Q T R G I T S F G S S T G C   157

62:    E T G Y P D G Y T R V T S Y L D W I E S   292
13:    E T G Y P D N Y T R V                     211
912:   E T G Y P D G Y T R V T S Y L D W I E S   294
31:    E T G Y P D G Y T R V T S Y L D W I E S   177

62:    N T G I A I D P                           300
912:   N T G I A I D P                           302
31:    N T G I A I D A *                         185
```

Figure 7 - Part 2

Amino Acid Comparison of Phim sequences

```
                1                                                  50
phim_p62        .......LLL ALVAA.ASAA EWRWQFRHPT VTPNPRAKNP FRVTKSSPVQ
phim_p912       .....IALLL ALVAATASAS EWRWQFRHPT VTPNPRAKNP FRPSKVAPVQ
phim5_1         PGRSRIALLL ALVAATASAS EWRWQFRHPT VTPNPRAKNP FRPSKVAPVQ
phim_p31        .......... .......... .......... .......... ..........

               51                                                 100
phim_p62        PPAVRGTKAV ENCGPVAPKN KIVGGMEVTP HAYPWQVGLF IDDMYFCGGS
phim_p912       PPAVRGTKAV ENCGQ.SKST KIVGGGEVTP HAYPWQVGLF IDDMYFCGGS
phim5_1         PPAVRGTKAV ENCGPVAPKN KIVGGQEVTP HAYPWQVGLF IDDMYFCGGS
phim_p31        .......... .......... .......... .......... ..........

               101                                                150
phim_p62        IISDEWVLTA AHCMDGAGFV EVVMGAHSIK DDTEATQVRA TSTDFFTHEN
phim_p912       IISEDWVLTA AHCMDGAGFV EVVMGAEKIH DDTEASRVSA ISTDFFTHEN
phim5_1         IISEDWVLTA AHCVDGAGFV EVVMGAHSIK DDTEASRISA TSIDFFTHEN
phim_p31        .......... .......... ...MGAHSIK DDTEASRVSA TSTDFFTHEN

               151                                                200
phim_p62        WNSFTLSNDL ALIKMPAPIE FNDVIQPVCL PTYTDASDDF VGESVTLTGW
phim_p912       WNSFLLTNDL ALIKMPAPIA FTDEIQPVCL PTYTDSDDDF VGESVTLTGW
phim5_1         WNSFTLTNDL ALIKMPAPIE FTPEIQFV.. .......... ..........
phim_p31        WNSFTLTNDL ALIKMPAPIE FTPEIQPVCL PSYTDAADDF IGESVVLTGW

               201                                                250
phim_p62        GKPSDSAFGI AEQLREVDVT TITTADCQAY YGIVTDKILC IDSEGGHGSC
phim_p912       GRASDSASGI SEVLREVDVT TITTADCQAY YGIVTDKILC IDSEGGHGSC
phim5_1         .......... .......... .......... .......... ..........
phim_p31        GRDSDAASGI SELLREVHVT TISTADCQAY YGIVTDKILC ISSEDGHGSC

               251                                                300
phim_p62        NGDSGGPMNY VTGGVTQTRG ITSFGSSTGC ETGYPDGYTR VTSYLDWIES
phim_p912       NGDSGGPMNY VTGGVTQTRG ITSFGSSTGC ETGYPDGYTR VTSYLDWIES
phim5_1         .......... .......... .......... .......... ..........
phim_p31        NGDSGGPMNY VTGGVTQTRG ITSFGSSTGC ETGYPDGYTR VTSYLDWIES

               301
phim_p62        NTGIAIDP
phim_p912       NTGIAIDP
phim5_1         ........
phim_p31        NTGIAIDA
```

Figure 8

SEQUENCE LISTING

<110> Franklin, Richard L.
Cowling, Didier S.P.
Hubbell, Jeffrey A.

<120> Treatment of Wounds

<130> 314572-103

<150> US 60/073,234
<151> 1998-02-23

<150> US 60/085,456
<151> 1998-05-14

<160> 52

<170> FastSEQ for Windows Version 3.0

<210> 1
<211> 943
<212> DNA
<213> Unknown

<400> 1
ctcttactcg cccttgtggc tgctgctagt gccgcagaat ggcgctggca gtttcgtcac
60
cctacagtga cccccaaccc tagggctaag aacccttca gagtcaccaa aagctctcca
120
gtccaaccac cagcagtcag aggaacaaag gctgttgaga actgtggacc agtagcacca
180
aggaacaaga ttgtaggagg catggaggtg actccccatg cttacccctg gcaggtggga
240
cttttcattg atgatatgta cttctgtggt ggatcaatca tctccgacga atgggtcctt
300
acagctgctc actgtatgga tggtgctggg tttgttgagg ttgtgatggg tgctcacagt
360
atccatgacg aaactgaggc cacacaggtc cgtgccacat caactgattt cttcacccac
420
gagaactgga actccttcac cctctccaat gatcttgctc tcattaagat gccagcacca
480
attgaattca acgatgtgat ccagcctgtc tgcctaccaa cctatactga tgctagtgat
540
gattttgttg gtgaatcagt cactcttact ggatggggta aaccatctga ctctgctttt
600
ggcatcgctg aacaacttcg tgaggttgat gtgacaacaa tcactactgc tgactgccag
660
gcatactacg gcattgtcac tgacaaaatc ctctgcatcg actccgaagg aggccatggt
720
tcctgcaatg gtgattccgg cgggccaatg aactatgtaa ctggtggtgt tactcagacc
780
cgtggtatta cctctttcgg atcctctacc ggctgcgaga ctggctaccc tgatggttac
840
acacgagtca ccagctatct ggactggatt gaatctaaca ctggcattgc cattgatcca
900
taaatacaat tctagcaaaa atacaataaa ttatacttaa atg
943

<210> 2
<211> 300
<212> PRT
<213> Panaeus vanameii

<400> 2

```
Leu Leu Leu Ala Leu Val Ala Ala Ala Ser Ala Ala Glu Trp Arg Trp
 1               5                  10                  15
Gln Phe Arg His Pro Thr Val Thr Pro Asn Pro Arg Ala Lys Asn Pro
            20                  25                  30
Phe Arg Val Thr Lys Ser Ser Pro Val Gln Pro Pro Ala Val Arg Gly
        35                  40                  45
Thr Lys Ala Val Glu Asn Cys Gly Pro Val Ala Pro Arg Asn Lys Ile
    50                  55                  60
Val Gly Gly Met Glu Val Thr Pro His Ala Tyr Pro Trp Gln Val Gly
65                  70                  75                  80
Leu Phe Ile Asp Asp Met Tyr Phe Cys Gly Gly Ser Ile Ile Ser Asp
                85                  90                  95
Glu Trp Val Leu Thr Ala Ala His Cys Met Asp Gly Ala Gly Phe Val
            100                 105                 110
Glu Val Val Met Gly Ala His Ser Ile His Asp Glu Thr Glu Ala Thr
        115                 120                 125
Gln Val Arg Ala Thr Ser Thr Asp Phe Phe Thr His Glu Asn Trp Asn
    130                 135                 140
Ser Phe Thr Leu Ser Asn Asp Leu Ala Leu Ile Lys Met Pro Ala Pro
145                 150                 155                 160
Ile Glu Phe Asn Asp Val Ile Gln Pro Val Cys Leu Pro Thr Tyr Thr
                165                 170                 175
Asp Ala Ser Asp Asp Phe Val Gly Glu Ser Val Thr Leu Thr Gly Trp
            180                 185                 190
Gly Lys Pro Ser Asp Ser Ala Phe Gly Ile Ala Glu Gln Leu Arg Glu
        195                 200                 205
Val Asp Val Thr Thr Ile Thr Thr Ala Asp Cys Gln Ala Tyr Tyr Gly
    210                 215                 220
Ile Val Thr Asp Lys Ile Leu Cys Ile Asp Ser Glu Gly Gly His Gly
225                 230                 235                 240
Ser Cys Asn Gly Asp Ser Gly Gly Pro Met Asn Tyr Val Thr Gly Gly
                245                 250                 255
Val Thr Gln Thr Arg Gly Ile Thr Ser Phe Gly Ser Ser Thr Gly Cys
            260                 265                 270
Glu Thr Gly Tyr Pro Asp Gly Tyr Thr Arg Val Thr Ser Tyr Leu Asp
        275                 280                 285
Trp Ile Glu Ser Asn Thr Gly Ile Ala Ile Asp Pro
    290                 295                 300
```

<210> 3
<211> 599
<212> DNA
<213> Unknown

<400> 3

```
gatgggtgct cacagtatcc atgacgatac tgaggcctct cgcgtcagtg ccacatcaac
60
tgatttcttc acccacgaga actggaactc cttcaccctc accaatgatc ttgctctcat
120
taagatgcca gcaccaattg aattcacacc tgaaattcaa cctgtctgcc taccaagcta
180
cactgatgct gctgatgatt tcattggtga atctgttgtc cttactggat ggggccgtga
240
ttctgatgct gcttccggca tctctgaact actccgtgag gttcatgtga ccacaatctc
300
```

cactgccgac tgccaggcat actacggcat tgtcactgac aaaatcctct gcatttcctc
360
tgaagacgga catggttctt gtaatggtga ttccggtggg ccaatgaact atgtaactgg
420
tggtgttact cagacccgtg gtattacctc cttcggatcc tctaccgggt gtgagactgg
480
ctaccctgat ggttacacac gtgtcaccag ctatctggac tggattgaat ctaacactgg
540
cattgccatt gatgcttgaa tataatacta gatatgtaat caaataaatt tcatgaatt
599

<210> 4
<211> 185
<212> PRT
<213> Panaeus vanameii

<400> 4

Met	Gly	Ala	His	Ser	Ile	His	Asp	Asp	Thr	Glu	Ala	Ser	Arg	Val	Ser
1			5					10					15		
Ala	Thr	Ser	Thr	Asp	Phe	Phe	Thr	His	Glu	Asn	Trp	Asn	Ser	Phe	Thr
		20					25					30			
Leu	Thr	Asn	Asp	Leu	Ala	Leu	Ile	Lys	Met	Pro	Ala	Pro	Ile	Glu	Phe
	35					40					45				
Thr	Pro	Glu	Ile	Gln	Pro	Val	Cys	Leu	Pro	Ser	Tyr	Thr	Asp	Ala	Ala
50					55					60					
Asp	Asp	Phe	Ile	Gly	Glu	Ser	Val	Val	Leu	Thr	Gly	Trp	Gly	Arg	Asp
65				70					75						80
Ser	Asp	Ala	Ala	Ser	Gly	Ile	Ser	Glu	Leu	Leu	Arg	Glu	Val	His	Val
			85					90					95		
Thr	Thr	Ile	Ser	Thr	Ala	Asp	Cys	Gln	Ala	Tyr	Tyr	Gly	Ile	Val	Thr
		100					105					110			
Asp	Lys	Ile	Leu	Cys	Ile	Ser	Ser	Glu	Asp	Gly	His	Gly	Ser	Cys	Asn
	115					120					125				
Gly	Asp	Ser	Gly	Gly	Pro	Met	Asn	Tyr	Val	Thr	Gly	Gly	Val	Thr	Gln
130					135					140					
Thr	Arg	Gly	Ile	Thr	Ser	Phe	Gly	Ser	Ser	Thr	Gly	Cys	Glu	Thr	Gly
145				150					155						160
Tyr	Pro	Asp	Gly	Tyr	Thr	Arg	Val	Thr	Ser	Tyr	Leu	Asp	Trp	Ile	Glu
			165					170					175		
Ser	Asn	Thr	Gly	Ile	Ala	Ile	Asp	Ala							
		180					185								

<210> 5
<211> 536
<212> DNA
<213> Unknown

<400> 5
cccgggcagg tccaggatcg ccctcttact tgcccttgtg gctgctacag ctagtgcttc
60
agaatggcgc tggcagttcc gtcaccccac tgtgaccccc aaccccagag ctaacaaccc
120
cttcagaccc agtaaagtcg ctccagtcca accaccagca gtcagaggaa caaaggctgt
180
tgagaactgt ggaccagtag caccaaagaa caagattgta ggagggcaag aagtgactcc
240
ccatgcttac ccctggcagg tgggactctt catcgatgac atgtacttct gcggtggatc
300
catcatctca gaggactggg tgcttacagc tgctcactgt gtggatggtg ctggttttgt
360
cgaagttgtg atgggtgctc acagtatcca tgacgatact gaggcctctc gcatcagtgc
420

cacatcaact gatttcttca cccacgagaa ctggaactcc ttcaccctca ccaatgatct
480
tgctctcatt aagatgccag cacccattga gttcacacct gaaattcaac ctgtct
536

<210> 6
<211> 178
<212> PRT
<213> Panaeus vanameii

<400> 6

```
Pro Gly Arg Ser Arg Ile Ala Leu Leu Leu Ala Leu Val Ala Ala Thr
 1               5                  10                  15
Ala Ser Ala Ser Glu Trp Arg Trp Gln Phe Arg His Pro Thr Val Thr
            20                  25                  30
Pro Asn Pro Arg Ala Asn Asn Pro Phe Arg Pro Ser Lys Val Ala Pro
        35                  40                  45
Val Gln Pro Pro Ala Val Arg Gly Thr Lys Ala Val Glu Asn Cys Gly
    50                  55                  60
Pro Val Ala Pro Lys Asn Lys Ile Val Gly Gly Gln Glu Val Thr Pro
65                  70                  75                  80
His Ala Tyr Pro Trp Gln Val Gly Leu Phe Ile Asp Asp Met Tyr Phe
                85                  90                  95
Cys Gly Gly Ser Ile Ile Ser Glu Asp Trp Val Leu Thr Ala Ala His
            100                 105                 110
Cys Val Asp Gly Ala Gly Phe Val Glu Val Val Met Gly Ala His Ser
        115                 120                 125
Ile His Asp Asp Thr Glu Ala Ser Arg Ile Ser Ala Thr Ser Thr Asp
    130                 135                 140
Phe Phe Thr His Glu Asn Trp Asn Ser Phe Thr Leu Thr Asn Asp Leu
145                 150                 155                 160
Ala Leu Ile Lys Met Pro Ala Pro Ile Glu Phe Thr Pro Glu Ile Gln
                165                 170                 175
Pro Val
```

<210> 7
<211> 633
<212> DNA
<213> Unknown

<400> 7

cacgcctacc cgtggcaggt gggacttttc attgatgata tgtacttctg tggaggatca
60
atcatctccg acgaatgggt ccttacagct gctcactgta tggatggtgc tggatttgtt
120
gaggttgtga tgggtgctca cagtatccat gacgaaactg aggccacaca ggtccgtgcc
180
acatcaactg atttcttcac acacgagaac tggaactcct tcaccctctc caatgatctt
240
gctctcatta agatgccagc accaattgaa ttcaacgatg tgatccagcc tgtctgccta
300
ccaacctata ctgatgccag tgatgatttt gttggtgaat cagtcactct tactggatgg
360
ggtaaaccat ctgactctgc ttttggcatc gctgaacaac ttcgtgaggt tgatgtgaca
420
acaatcacta ctgctgactg ccaggcatac tacggcattg tcactgacaa aatcctctgc
480
atcgactccg aaggaggcca tggttcctgc aatggtgatt ccggtgggcc aatgaactat
540
gtaactggtg gtgttactca gacccgtggt attaccttct tcggatcctc taccggctgc
600

gagactgggt accctgataa ttacacacga gtc
633

<210> 8
<211> 211
<212> PRT
<213> Panaeus vanameii

<400> 8

```
His Ala Tyr Pro Trp Gln Val Gly Leu Phe Ile Asp Asp Met Tyr Phe
 1               5                  10                  15
Cys Gly Gly Ser Ile Ile Ser Asp Glu Trp Val Leu Thr Ala Ala His
            20                  25                  30
Cys Met Asp Gly Ala Gly Phe Val Glu Val Val Met Gly Ala His Ser
        35                  40                  45
Ile His Asp Glu Thr Glu Ala Thr Gln Val Arg Ala Thr Ser Thr Asp
    50                  55                  60
Phe Phe Thr His Glu Asn Trp Asn Ser Phe Thr Leu Ser Asn Asp Leu
65                  70                  75                  80
Ala Leu Ile Lys Met Pro Ala Pro Ile Glu Phe Asn Asp Val Ile Gln
                85                  90                  95
Pro Val Cys Leu Pro Thr Tyr Thr Asp Ala Ser Asp Asp Phe Val Gly
            100                 105                 110
Glu Ser Val Thr Leu Thr Gly Trp Gly Lys Pro Ser Asp Ser Ala Phe
        115                 120                 125
Gly Ile Ala Glu Gln Leu Arg Glu Val Asp Val Thr Thr Ile Thr Thr
    130                 135                 140
Ala Asp Cys Gln Ala Tyr Tyr Gly Ile Val Thr Asp Lys Ile Leu Cys
145                 150                 155                 160
Ile Asp Ser Glu Gly Gly His Gly Ser Cys Asn Gly Asp Ser Gly Gly
                165                 170                 175
Pro Met Asn Tyr Val Thr Gly Gly Val Thr Gln Thr Arg Gly Ile Thr
            180                 185                 190
Ser Phe Gly Ser Ser Thr Gly Cys Glu Thr Gly Tyr Pro Asp Asn Tyr
        195                 200                 205
Thr Arg Val
    210
```

<210> 9
<211> 967
<212> DNA
<213> Unknown

<400> 9

atcgccctct tactcgccct tgtggctgcc actgctagtg cttcagaatg gcgctggcag
60
ttccgtcacc ccaccgtgac ccccaacccc agagctaaca acccccttcag accaagtaaa
120
gttgctccag tccaaccacc agcagtcaga ggaacaaagg ctgtacccaa ctgtggacag
180
tcaaagtcta ccaagattgt aggaggtggt gaggtaactc cccatgctta cccctggcag
240
gtgggacttt tcattgatga catgtacttc tgcggkggat ccatcatctc agaggactgg
300
gtccttacag ctgctcactg tatggatggt gctgggtttg ttgaggttgt gatgggtgct
360
cacaagatcc atgatgatac tgaggcctct cgcgtcagtg ccatatcaac tgatttcttc
420
acccacgaga actggaactc cttccttctc accaatgatc ttgctctcat taagatgcca
480
gcacccattg cattcactga tgagatccag cctgtatgcc tgccaaccta cactgactcc
540

gatgatgatt ttgttggtga atcagtcact cttactggct ggggtcgtgc atctgactct
600
gctagcggca tctctgaagt acttcgtgag gttgatgtga caacaataac tactgccgac
660
tgccaggcat actatggtat tgtcactgac aaaatcctct gcatcgactc agaaggaggt
720
catgggtctt gcaatggtga ttccggtggg ccaatgaact atgtaactgg tggtgttact
780
cagacccgtg gtattacctc cttcggatcc tctaccggct gtgagactgg ctaccctgat
840
ggttacacac gagtcaccag ctatctagac tggattgaat ctaacactgg cattgccatt
900
gatccttgaa taatattcta gctgaatgat aataaattca tgattgataa tcaaaaaaaa
960
aaaaaaa
967

<210> 10
<211> 302
<212> PRT
<213> Panaeus vanameii

<400> 10

Ile	Ala	Leu	Leu	Leu	Ala	Leu	Val	Ala	Ala	Thr	Ala	Ser	Ala	Ser	Glu
1				5					10					15	
Trp	Arg	Trp	Gln	Phe	Arg	His	Pro	Thr	Val	Thr	Pro	Asn	Pro	Arg	Ala
			20					25					30		
Asn	Asn	Pro	Phe	Arg	Pro	Ser	Lys	Val	Ala	Pro	Val	Gln	Pro	Pro	Ala
		35					40					45			
Val	Arg	Gly	Thr	Lys	Ala	Val	Pro	Asn	Cys	Gly	Gln	Ser	Lys	Ser	Thr
	50					55					60				
Lys	Ile	Val	Gly	Gly	Gly	Glu	Val	Thr	Pro	His	Ala	Tyr	Pro	Trp	Gln
65					70					75					80
Val	Gly	Leu	Phe	Ile	Asp	Asp	Met	Tyr	Phe	Cys	Gly	Gly	Ser	Ile	Ile
				85					90					95	
Ser	Glu	Asp	Trp	Val	Leu	Thr	Ala	Ala	His	Cys	Met	Asp	Gly	Ala	Gly
			100					105					110		
Phe	Val	Glu	Val	Val	Met	Gly	Ala	His	Lys	Ile	His	Asp	Asp	Thr	Glu
		115					120					125			
Ala	Ser	Arg	Val	Ser	Ala	Ile	Ser	Thr	Asp	Phe	Phe	Thr	His	Glu	Asn
	130					135					140				
Trp	Asn	Ser	Phe	Leu	Leu	Thr	Asn	Asp	Leu	Ala	Leu	Ile	Lys	Met	Pro
145					150					155					160
Ala	Pro	Ile	Ala	Phe	Thr	Asp	Glu	Ile	Gln	Pro	Val	Cys	Leu	Pro	Thr
				165					170					175	
Tyr	Thr	Asp	Ser	Asp	Asp	Asp	Phe	Val	Gly	Glu	Ser	Val	Thr	Leu	Thr
			180					185					190		
Gly	Trp	Gly	Arg	Ala	Ser	Asp	Ser	Ala	Ser	Gly	Ile	Ser	Glu	Val	Leu
		195					200					205			
Arg	Glu	Val	Asp	Val	Thr	Thr	Ile	Thr	Thr	Ala	Asp	Cys	Gln	Ala	Tyr
	210					215					220				
Tyr	Gly	Ile	Val	Thr	Asp	Lys	Ile	Leu	Cys	Ile	Asp	Ser	Glu	Gly	Gly
225					230					235					240
His	Gly	Ser	Cys	Asn	Gly	Asp	Ser	Gly	Gly	Pro	Met	Asn	Tyr	Val	Thr
				245					250					255	
Gly	Gly	Val	Thr	Gln	Thr	Arg	Gly	Ile	Thr	Ser	Phe	Gly	Ser	Ser	Thr
			260					265					270		
Gly	Cys	Glu	Thr	Gly	Tyr	Pro	Asp	Gly	Tyr	Thr	Arg	Val	Thr	Ser	Tyr
		275					280					285			
Leu	Asp	Trp	Ile	Glu	Ser	Asn	Thr	Gly	Ile	Ala	Ile	Asp	Pro		
	290					295					300				

<210> 11
<211> 535
<212> DNA
<213> Unknown

<220>
<221> CDS
<222> (2)...(235)

<400> 11
c ccg ggc agg tcc agg atc gcc ctc tta ctt gcc ctt gtg gct gct aca
49
Pro Gly Arg Ser Arg Ile Ala Leu Leu Leu Ala Leu Val Ala Ala Thr
1 5 10 15

gct agt gct tca gaa tgg cgc tgg cag ttc cgt cac ccc act gtg acc
97
Ala Ser Ala Ser Glu Trp Arg Trp Gln Phe Arg His Pro Thr Val Thr
20 25 30

ccc aac ccc aga gct aac aac ccc ttc aga ccc agt aaa gtc gct cca
145
Pro Asn Pro Arg Ala Asn Asn Pro Phe Arg Pro Ser Lys Val Ala Pro
35 40 45

gtt caa cca cca gca gtc aga gga aca aag gct gtt gag aac tgt gga
193
Val Gln Pro Pro Ala Val Arg Gly Thr Lys Ala Val Glu Asn Cys Gly
50 55 60

cca gta gca cca aag aac aag att gta gga ggg caa gaa gtg
235
Pro Val Ala Pro Lys Asn Lys Ile Val Gly Gly Gln Glu Val
65 70 75

actccccatg cttacccctg gcaggtggga ctcttcatcg atgacatgta cttcttcggt
295
ggatccatca tctcagagga ctgggtcgtt acagctcgtc actgtatgga tggtcgtggt
355
tttgtcgaag ttgtgatggg tgctcacagt atcctagacg atactgaggc ctctcgcatg
415
agtgccacat caactgattt cttcacccac gagaactgga actccttcac cctcaccaat
475
gatcttgctc tcattaagat gccagcaccc attgagttca cacctgaaat tcaacctgtc
535

<210> 12
<211> 78
<212> PRT
<213> Unknown

<400> 12
Pro Gly Arg Ser Arg Ile Ala Leu Leu Leu Ala Leu Val Ala Ala Thr
1 5 10 15
Ala Ser Ala Ser Glu Trp Arg Trp Gln Phe Arg His Pro Thr Val Thr
20 25 30
Pro Asn Pro Arg Ala Asn Asn Pro Phe Arg Pro Ser Lys Val Ala Pro
35 40 45
Val Gln Pro Pro Ala Val Arg Gly Thr Lys Ala Val Glu Asn Cys Gly
50 55 60
Pro Val Ala Pro Lys Asn Lys Ile Val Gly Gly Gln Glu Val
65 70 75

<210> 13
<211> 178
<212> PRT
<213> Panaeus vanameii

<400> 13
Pro Gly Arg Ser Arg Ile Ala Leu Leu Leu Ala Leu Val Ala Ala Thr
1 5 10 15
Ala Ser Ala Ser Glu Trp Arg Trp Gln Phe Arg His Pro Thr Val Thr
20 25 30
Pro Asn Pro Arg Ala Asn Asn Pro Phe Arg Pro Ser Lys Val Ala Pro
35 40 45
Val Gln Pro Pro Ala Val Arg Gly Thr Lys Ala Val Glu Asn Cys Gly
50 55 60
Pro Val Ala Pro Lys Asn Lys Ile Val Gly Gly Gln Glu Val Thr Pro
65 70 75 80
His Ala Tyr Pro Trp Gln Val Gly Leu Phe Ile Asp Asp Met Tyr Phe
85 90 95
Phe Gly Gly Ser Ile Ile Ser Glu Asp Trp Val Val Thr Ala Arg His
100 105 110
Cys Met Asp Gly Arg Gly Phe Val Glu Val Val Met Gly Ala His Ser
115 120 125
Ile Leu Asp Asp Thr Glu Ala Ser Arg Met Ser Ala Thr Ser Thr Asp
130 135 140
Phe Phe Thr His Glu Asn Trp Asn Ser Phe Thr Leu Thr Asn Asp Leu
145 150 155 160
Ala Leu Ile Lys Met Pro Ala Pro Ile Glu Phe Thr Pro Glu Ile Gln
165 170 175
Pro Val

<210> 14
<211> 25
<212> PRT
<213> Panaeus vanameii

<400> 14
Ile Val Gly Gly Val Glu Ala Thr Pro His Ser Trp Pro His Gln Ala
1 5 10 15
Ala Leu Phe Ile Asp Asp Met Tyr Phe
20 25

<210> 15
<211> 20
<212> PRT
<213> Panaeus vanameii

<220>
<221> VARIANT
<222> (1)...(20)
<223> Xaa = Any Amino Acid

<400> 15
Ile Val Gly Gly Val Glu Ala Thr Pro His Ser Xaa Pro His Gln Ala
1 5 10 15
Ala Leu Phe Ile
20

<210> 16
<211> 25
<212> PRT

<213> Panaeus monodon tryptic

<400> 16
Ile Val Gly Gly Thr Ala Val Thr Pro Gly Glu Phe Pro Tyr Gln Leu
1 5 10 15
Ser Phe Gln Asp Ser Ile Glu Gly Val
20 25

<210> 17
<211> 25
<212> PRT
<213> Panaeus monodon chymotryptic

<400> 17
Ile Val Gly Gly Val Glu Ala Val Pro Gly Val Trp Pro Tyr Gln Ala
1 5 10 15
Ala Leu Phe Ile Ile Asp Met Tyr Phe
20 25

<210> 18
<211> 25
<212> PRT
<213> Panaeus monodon chymotryptic

<400> 18
Ile Val Gly Gly Val Glu Ala Val Pro His Ser Trp Pro Tyr Gln Ala
1 5 10 15
Ala Leu Phe Ile Ile Asp Met Tyr Phe
20 25

<210> 19
<211> 25
<212> PRT
<213> Uca pugilator enzyme

<400> 19
Ile Val Gly Gly Val Glu Ala Val Pro Asn Ser Trp Pro His Gln Ala
1 5 10 15
Ala Leu Phe Ile Asp Asp Met Tyr Phe
20 25

<210> 20
<211> 20
<212> PRT
<213> Uca pugilator enzyme

<400> 20
Ile Val Gly Gly Gln Asp Ala Thr Pro Gly Gln Phe Pro Tyr Gln Leu
1 5 10 15
Ser Phe Gln Asp
20

<210> 21
<211> 20
<212> PRT
<213> Kamchatka crab

<220>
<221> VARIANT
<222> (1)...(20)
<223> Xaa = Any Amino Acid

<400> 21
Ile Val Gly Gly Gln Glu Ala Ser Pro Gly Ser Trp Pro Xaa Gln Val
1 5 10 15
Gly Leu Phe Phe
20

<210> 22
<211> 20
<212> PRT
<213> Kamchatka crab

<400> 22
Ile Val Gly Gly Thr Glu Val Thr Pro Gly Glu Ile Pro Tyr Gln Leu
1 5 10 15
Ser Leu Gln Asp
20

<210> 23
<211> 20
<212> PRT
<213> Kamchatka crab

<400> 23
Ile Val Gly Gly Thr Glu Val Thr Pro Gly Glu Ile Pro Tyr Gln Leu
1 5 10 15
Ser Phe Gln Asp
20

<210> 24
<211> 20
<212> PRT
<213> Kamchatka crab

<400> 24
Ile Val Gly Gly Ser Glu Ala Thr Ser Gly Gln Phe Pro Tyr Gln Xaa
1 5 10 15
Ser Phe Gln Asp
20

<210> 25
<211> 20
<212> PRT
<213> Crayfish protease

<400> 25
Ile Val Gly Gly Thr Asp Ala Thr Leu Gly Glu Phe Pro Tyr Gln Leu
1 5 10 15
Ser Phe Gln Asn
20

<210> 26
<211> 25
<212> PRT
<213> Salmon enzyme

<400> 26
Ile Val Gly Gly Tyr Glu Cys Lys Ala Tyr Ser Gln Ala Tyr Gln Val
1 5 10 15
Ser Leu Asn Ser Gly Tyr His Tyr Cys
20 25

<210> 27

<211> 25
<212> PRT
<213> Atlantic cod

<400> 27
Ile Val Gly Gly Tyr Glu Cys Thr Lys His Ser Gln Ala His Gln Val
1 5 10 15
Ser Leu Asn Ser Gly Tyr His Tyr Cys
20 25

<210> 28
<211> 25
<212> PRT
<213> Atlantic cod

<400> 28
Ile Val Gly Gly Tyr Glu Cys Thr Arg His Ser Gln Ala His Gln Val
1 5 10 15
Ser Leu Asn Ser Gly Tyr His Tyr Cys
20 25

<210> 29
<211> 11
<212> PRT
<213> Unknown

<400> 29
Leu Leu Leu Ala Leu Val Ala Ala Ala Ser Ala
1 5 10

<210> 30
<211> 19
<212> PRT
<213> Unknown

<400> 30
Pro Gly Arg Ser Arg Ile Ala Leu Leu Leu Ala Leu Val Ala Ala Thr
1 5 10 15
Ala Ser Ala

<210> 31
<211> 52
<212> PRT
<213> Unknown

<400> 31
Ala Glu Trp Arg Trp Gln Phe Arg His Pro Thr Val Thr Pro Asn Pro
1 5 10 15
Arg Ala Lys Asn Pro Phe Arg Val Thr Lys Ser Ser Pro Val Gln Pro
20 25 30
Pro Ala Val Arg Gly Thr Lys Ala Val Glu Asn Cys Gly Pro Val Ala
35 40 45
Pro Arg Asn Lys
50

<210> 32
<211> 52
<212> PRT
<213> Unknown

<400> 32

Ser Glu Trp Arg Trp Gln Phe Arg His Pro Thr Val Thr Pro Asn Pro
1 5 10 15
Arg Ala Asn Asn Pro Phe Arg Pro Ser Lys Val Ala Pro Val Gln Pro
20 25 30
Pro Ala Val Arg Gly Thr Lys Ala Val Glu Asn Cys Gly Pro Val Ala
35 40 45
Pro Lys Asn Lys
50

<210> 33
<211> 11
<212> PRT
<213> Unknown

<400> 33
Ala Val Glu Asn Cys Gly Pro Val Ala Pro Arg
1 5 10

<210> 34
<211> 13
<212> PRT
<213> Unknown

<400> 34
Ala Val Glu Asn Cys Gly Pro Val Ala Pro Arg Asn Lys
1 5 10

<210> 35
<211> 14
<212> PRT
<213> Unknown

<400> 35
Gly Thr Lys Ala Val Glu Asn Cys Gly Pro Val Ala Pro Arg
1 5 10

<210> 36
<211> 16
<212> PRT
<213> Unknown

<400> 36
Gly Thr Lys Ala Val Glu Asn Cys Gly Pro Val Ala Pro Arg Asn Lys
1 5 10 15

<210> 37
<211> 24
<212> PRT
<213> Unknown

<400> 37
Ser Ser Pro Val Gln Pro Pro Ala Val Arg Gly Thr Lys Ala Val Glu
1 5 10 15
Asn Cys Gly Pro Val Ala Pro Arg
20

<210> 38
<211> 26
<212> PRT
<213> Unknown

<400> 38

Ser Ser Pro Val Gln Pro Pro Ala Val Arg Gly Thr Lys Ala Val Glu
1 5 10 15
Asn Cys Gly Pro Val Ala Pro Arg Asn Lys
20 25

<210> 39
<211> 9
<212> PRT
<213> Unknown

<400> 39
Ala Val Glu Asn Cys Gly Pro Val Ala
1 5

<210> 40
<211> 20
<212> PRT
<213> Unknown

<400> 40
Met Pro Gly Arg Ser Arg Ile Ala Leu Leu Leu Ala Leu Val Ala Ala
1 5 10 15
Thr Ala Ser Ala
20

<210> 41
<211> 21
<212> PRT
<213> Unknown

<220>
<221> VARIANT
<222> (1)...(21)
<223> Xaa = Any Amino Acid

<400> 41
Met Xaa Pro Gly Arg Ser Arg Ile Ala Leu Leu Leu Ala Leu Val Ala
1 5 10 15
Ala Thr Ala Ser Ala
20

<210> 42
<211> 5
<212> PRT
<213> Unknown

<400> 42
Asp Asp Asp Asp Lys
1 5

<210> 43
<211> 16
<212> DNA
<213> Unknown

<400> 43
cacgcctacc ctggca
16

<210> 44
<211> 21
<212> DNA

<213> Unknown

<400> 44
gtgttggact cgatccagat c
21

<210> 45
<211> 4
<212> PRT
<213> Unknown

<400> 45
Ser Arg Ile Ala
1

<210> 46
<211> 5
<212> PRT
<213> Unknown

<400> 46
Arg Ser Arg Ile Ala
1 5

<210> 47
<211> 6
<212> PRT
<213> Unknown

<400> 47
Gly Arg Ser Arg Ile Ala
1 5

<210> 48
<211> 7
<212> PRT
<213> Unknown

<400> 48
Pro Gly Arg Ser Arg Ile Ala
1 5

<210> 49
<211> 19
<212> PRT
<213> Uknown

<400> 49
Met Pro Gly Arg Ser Arg Ile Ala Leu Leu Leu Ala Leu Val Ala Ala
1 5 10 15
Ala Ser Ala

<210> 50
<211> 21
<212> PRT
<213> Unknown

<220>
<221> VARIANT
<222> (1)...(21)
<223> Xaa = Any Amino Acid

<400> 50
Met Xaa Pro Gly Arg Ser Arg Ile Ala Leu Leu Leu Ala Leu Val Leu
1 5 10 15
Ala Ala Ala Ser Ala
20

<210> 51
<211> 25
<212> PRT
<213> Unknown

<400> 51
Met Pro Gly Arg Ser Arg Ile Ala Leu Leu Leu Ala Leu Val Ala Ala
1 5 10 15
Thr Ala Ser Ala Ser Glu Trp Arg Trp
20 25

<210> 52
<211> 24
<212> PRT
<213> Unknown

<400> 52
Met Pro Gly Arg Ser Arg Ile Ala Leu Leu Leu Ala Leu Val Ala Ala
1 5 10 15
Ala Ser Ala Ala Glu Trp Arg Trp
20